MULTIPLE NUTRIENT ADAPTIVE MECHANISMS AFFECT ASPARAGINASE RESISTANCE IN MOLT-4 HUMAN LEUKEMIA CELLS

By

ARA M. ASLANIAN

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This thesis is dedicated to Oscar and Lucy Aslanian

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3-MG	3-O-methyl glucose
4eBP1	eukaryotic initiation factor 4e binding protein 1
4F2hc	4F2 heavy chain
AARE	amino acid response element
AAT	aspartate aminotransferase
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
AMP	adenosine monophosphate
AOA	aminooxyacetate
AP-1	activating protein 1
AS	asparagine synthetase
ASNase	asparaginase
ATCC	American type culture collection
ATF	activating transcription factor
ATP	adenosine triphosphate
AZC	azetidine-2-carboxylate
BCH	2-aminobicyclo(2,2,1)-heptane 2-carboxylic acid
BHK	baby hamster kidney
BSA	bovine serum albumin

C/EBP	CCAAT/ehancer binding protein
cAMP	cyclic AMP
CAT	chloramphenicol acetyl transferase
CBP	CREB-binding protein
CDK	cyclin dependent kinase
cDNA	complementary DNA
CHOP	C/EBP homologous protein
CLL	chronic lymphoblastic leukemia
CNS	central nervous system
CP	creatine phoshpate
CPK	creatine phosphokinase
CPM	counts per minute
CR	complete remission
CRE	cAMP response element
CREB	CRE binding protein
CTP	cytidine triphosphate
DMSO	dimethyl sulfoxide
DN	dominant negative
DPM	disentigrations per minute
EDTA	ethylenediamine-tetraacetic acid
EH	Eps homology
eIF	eukaryotic initiation factor
Elk-1	Ets related protein kinase 1

EMS	ethyl methane sulfonate
EMSA	electromobility shift assay
EOR	ER overload response
ER	endoplasmic reticulum
ERK1/2	extracellular regulated kinases 1/2
ERN1	ER to nucleus signaling
ERSE	endoplasmic reticulum stress element
EST	expressed sequence tag
FBS	fetal bovine serum
FIRE	*fos* intragenic regulatory element
FITC	flourescein isothiocyanate
FRAP	FK506 binding protein
G6PD	glucose-6-phosphate dehydrogenase
GABA	γ-isobutyric acid
GADD	growth arrest and DNA damage
GAP	GTPase activating protein
GCD	general control derepressable
GCN	general control nonderepressable
GDP	guanidine diphosphate
GFP	green fluorescent protein
GNEF	guanine nucleotide exchange factor
GRE	glucocorticoid responsive element
GRP	glucose regulated protein

GS	glutamine synthetase
GSH	glutathione
GTP	guanidine triphosphate
HAC1	homologous to ATF and CREB 1
HEK	human embryonic kidney
HEPES	N-2-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid
HisRS	hystidyl-tRNA synthetases
HRP	horseradish peroxidase
HSP27	heat shock protein 27
IκB	inhibitory κB
IKK	IκB kinase
IRE1	inositol requiring element 1
IRES	internal ribosome entry site
IU	international unit
JNK	c-*jun* N-terminal kinase
JRS	Jensen rat sarcoma
Km	Michaelis constant
KRP	Kreb's ringer phosphate
MAPK	mitogen activated protein kinase
MAPKAPK2	MAPK activating protein kinase 2
MBF1	multiprotein bridging factor 1
MDR1	multidrug resistance 1
MEK	extracellular regulated kinase

MEKK	MEK kinase
MKK	MEK kinase kinase
MOPS	3-(N-morpholino) ethanesulfonic acid
MSO	methionine sulfoximine
mTOR	mammalian target of rapamycin
MTT	3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor κB
NIK	NF-κB-inducing kinase
OA	okadaic acid
ODC	ornithine decarboxylase
ORF	open reading frame
PAK	p21-activated kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PEPCK	phosphhoenolpyruvate carboxy kinase
PERK	pancreatic eIF2α kinase
PH	partial hepatectomy
PHA	phytohemagglutinin
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
Ral	Ras related

RalBP1	Ral binding protein 1
Reps1	RalBP1 associated Eps-homology domain protein 1
Rlg1	tRNA ligase
ROI	reactive oxygen species
Rsk	ribosomal S6 kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase PCR
S6	ribosomal protein S6
S6K	S6 kinase
SAPK	stress activated protein kinase
SD	standard deviation
SDB	sample dilution buffer
SDS	sodium dodecyl sulfate
SEK	stress/extracellular regulated kinases
SHC3	Src homology domain 3
Sos	son of sevenless
SRE	serum response element
SV40	simian virus 40
TBP	TATA binding protein
TCR	T-cell receptor
TdT	terminal deoxynucleotidyl transferase
TIP	transport inactivating protein
TMB-8	3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester

TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
UPR	unfolded protein response
UPRE	UPR element
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium

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By

Ara M. Aslanian

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Chairman: Michael S. Kilberg, Ph. D.
Major Department: Biochemistry and Molecular Biology

Asparaginase (ASNase) is a component of most current treatment protocols for childhood acute lymphoblastic leukemia (ALL). The basis of this therapy is thought to be leukemia cell sensitivity to asparagine starvation because of insufficient asparagine synthetase (AS) enzyme activity. However, in many patients ASNase resistance develops, most likely due to the selection of cells with increased AS expression and perhaps other unknown metabolic perturbations. The transcriptional regulation of AS after ASNase treatment and amino acid starvation was investigated in the human ALL cell line, MOLT-4. Parental MOLT-4 leukemia cells expressed a low level of AS, but were capable of inducing AS mRNA and protein expression in response to amino acid deprivation. Asparaginase resistant cells maintained a much higher level of AS expression that was only partially reversible, suggesting that resistance may be a stable phenotype. In addition, overexpression of AS in sensitive MOLT-4 cells afforded protection from ASNase-induced cytotoxicity.

Given that ASNase resistant cells express high levels of AS, it was of interest to determine whether enhanced availability of the AS substrates, aspartate and glutamine, also contributes to ASNase resistance. The neutral amino acid transport Systems A and ASC were up-regulated in ASNase resistant cells, whereas anionic amino acid transport systems are not expressed in MOLT-4 cells. Furthermore, transport via System A was essential for complete ASNase resistance.

In terms of intracellular synthesis, glutamine production was necessary to maintain cell viability at low extracellular glutamine levels, and for complete ASNase resistance. Cellular synthesis of aspartate, as opposed to transport, was important for both parental and resistant cells.

To examine the signaling pathways of amino acid-dependent gene regulation, ASNase induced AS expression was tested in the presence of specific signal transduction pathway inhibitors. Blockade of the mammalian target of rapamycin (mTOR) and extracellular regulated kinases 1 and 2 (ERK1/2) pathways resulted in reduced AS expression during amino acid, but not glucose starvation. Collectively, these studies provide evidence that both substrate availability and increased AS enzymatic activity contribute to ASNase resistance in childhood ALL, and resistant cells use both reversible and irreversible nutrient adaptive mechanisms to maintain viability in the presence of the drug.

CHAPTER 1
INTRODUCTION

The leukemias are a group of disorders of the blood forming cells characterized by the unregulated proliferation of leukocytes. Acute lymphoblastic leukemia (ALL) is distinguished by the presence of immature malignant lymphocytes of either B- or T- cell origin. The rate of the disease is approximately four per 100,000 per year, and has a peak of incidence at 3 to 4 years of age (Pierce et al., 1969; Fraumeni et al., 1967). Currently, ALL represents approximately 40% of all leukemias diagnosed in the United States, and is the most common malignancy in children, representing 25% of all pediatric cancers (Margolin et al., 1997). Acute lymphoblastic leukemia occurs most frequently in industrialized countries, consistent with the hypothesis that the initial transforming event can be initiated by exposure to toxic chemicals and radiation (Folley et al., 1952)

Childhood ALL is caused by the malignant expansion of a single progenitor lymphocyte and subsequent infiltration of the bloodstream and organs by immature lymphoblasts. The clonal nature of ALL can be demonstrated by the observation that the X-linked gene glucose-6-phosphate dehydrogenase (G6PD) is present in one isoform in the leukemic cell population of lymphoblasts from females who, under normal circumstances, have two G6PD polymorphisms (Dow et al., 1985). The high frequency of specific mutations in particular subtypes of leukemia indicates that discrete changes in the progenitor cell give rise to the malignancy. Currently, over 100 chromosomal translocations, breakages, and differences in ploidy have been identified for various leukemias. In ALL cases alone, over 20 different chromosomal translocations have been

identified that recur in a nonrandom manner (Raimondi, 1993). In addition to chromosomal abnormalities, ALL may be distinguished by immunophenotyping the differentiation antigens displayed on B- and T- lineage cells. CD19 and CD22, present in early B-cell development, are universal markers for B-cell ALL (Campana et al., 1985), while T- cell ALL may be identified by early stage T-cell receptor (TCR) rearrangement and terminal deoxynucleotidyl transferase (TdT) activity (Felix et al., 1991). The classification of ALL cases based on these cytogenetic and morphologic characteristics will often dictate the mode and intensity of treatment.

The history of the treatment of childhood acute lymphoblastic leukemia (ALL) is a success of cancer therapy, and serves as a model for the application of modern research to the improvement of patient care. In 1945, patients presenting with acute forms of leukemia rarely were expected to live more than a few months. With the advent of single-agent chemotherapy in 1950, and combination chemotherapy in the mid-1950s, 5-year survival rates for ALL achieved levels of approximately 30%. The recognition that many leukemic blastocysts achieve sanctuary in the spinal cord led to the introduction of central nervous system (CNS) prophylaxis in the late 1960s, which, in combination with intensive induction therapy and an effective continuation regimen, improved complete remission (CR) rates to approximately 50% by 1980.

Currently, the most effective means of treating childhood ALL is a rapid, intense "remission induction" regimen to quickly clear the body of leukemic clones. When used alone, ASNase induces remission in 63% of patients (Nesbit et al., 1981), however, the primary remission induction therapy often includes a course of the anti-metabolite vincristine and the cortical steroid prednisone. When these agents are used alone, relapse

occurs quickly in many patients. However, the addition of ASNase to the induction regimen increases the remission rate to approximately 95% (Sanz et al., 1986). Also, the event-free survival rates over a period of 8 years are enhanced approximately 28% when ASNase is added to existing protocols (Koizumi and Fujimoto, 1984), making this drug an effective agent when used as part of a comprehensive treatment plan for ALL. Asparaginase, which catalyzes the hydrolysis of asparagine to aspartate and ammonia, occupies a unique position in cancer chemotherapy because it is the only agent that does not require direct contact and transport into the target cell. Instead, the mode of action is a reduction of the circulating levels of asparagine, which is selectively toxic to neoplastic lymphocytes. Asparaginase appears to be most effective against ALL, with a reduction in peripheral blood blast number, organomegaly, and lymph node mass within 1 to 2 weeks of administration. There also have been isolated reports of ASNase effectiveness against acute myeloblastic leukemia, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and chronic myelogenous leukemia. However, the responses to these malignancies generally is not considered as favorable as that for ALL (Capizzi and Holcenberg, 1993). More recently, there has been a report on the use of ASNase in the treatment of AIDS-related lymphoma (Tuple, 1998)

The development of ASNase as an anti-tumor agent is an example of a fortuitous discovery followed by a series of experiments that carefully refined the initial observations, eventually leading to the modern ASNase treatment regimen. Originally intending to use a preparation of rabbit antilymphoma antibodies to treat tumor-bearing mice, Kidd (1953) discovered that the guinea pig serum used as a source of complement was alone sufficient to cause tumor regression. Considering the observation by Clementi

(1922) that guinea pig serum was the only animal sera capable of hydrolyzing asparagine, Broome (1961) demonstrated conclusively that ASNase was the agent responsible for the antilymphoma effect of guinea pig serum. In 1964, Mashburn and Wriston showed that ASNase isolated from *E. coli* was equally as effective as guinea pig serum in promoting tumor regression, which led to the large-scale production of the bacterial enzyme as an oncolytic agent for humans (Mashburn and Wriston, 1964).

Asparaginase, which catalyzes the hydrolysis of asparagine to aspartic acid and ammonia, has been found in gram negative bacteria, mycobacteria, yeasts, molds, plants, and vertebrates, however the most clinically useful enzyme is the Type II isozyme located in the periplasmic space of *E. coli* (Wriston and Yellin, 1973; Wriston 1985; Yurek, 1983). The enzyme is a 141,000-dalton protein comprising four identical subunits, each containing an active site (Jennings and Beacham, 1990; Maita et al., 1980). The effectiveness of the *E. coli* preparation is due primarily to the low (12 μM) Km for asparagine (Cedar and Schwartz, 1967), and the relatively low glutaminase side reaction as compared to the *Erwinia chrysanthemi* ASNase (0.03 vs. 0.10 glutaminase/ASNase ratio), limiting the immunosuppressive side effects associated with glutamine deprivation. In addition to its favorable catalytic properties, the *E. coli* enzyme also has a relatively long *in vivo* half-life. The commercially available Merck Elspar preparation has an average half-life of 23 h; almost double that of the *Erwinia* enzyme, which typically averages 10 h (Capizzi and Holcenberg, 1993). The importance of the *in vivo* clearance rate on enzyme efficacy is demonstrated by the fact that ASNases from *Bacillus coagulans*, *Fusarium tricinctum*, and *Candida albicans* are cleared from the

bloodstream within 30 min to 1 hr and have no anti-tumor activity (Chabner and Loo, 1996).

The typical dosage of ASNase delivered to the patient is 6000 IU/m^2, injected intramuscularly every other day, resulting in an immediate decrease in circulating asparagine to below detectable levels that is maintained for up to a week after the discontinuation of therapy (Ohnuma et al., 1970). The glutaminase side reaction of *E. coli* ASNase also produces an immediate decrease in the plasma glutamine levels in the patient, however, the glutamine concentration typically rises after five days of treatment, presumably because of the induction of intracellular glutamine synthetases (Miller et al., 1969).

Under normal circumstances, the plasma level of asparagine in mammals is controlled by the liver, which contains ASNase and AS activities (EC 6.3.5.4) to maintain plasma homeostasis (Woods and Handschumacher, 1971). Asparagine synthetase catalyzes the formation of asparagine from aspartate, using glutamine as the amido nitrogen donor, with the concomitant hydrolysis of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and pyrophosphate. During ASNase treatment, the level of circulating ASNase outstrips the ability of the liver to maintain normal asparagine levels, thus protein synthesis is disrupted in affected tissues. The pancreas also is an asparagine sanctuary, having high levels of AS, however, this organ cannot return asparagine to the bloodstream during asparagine deprivation (Milman et al., 1979).

The antineoplastic activity of ASNase appears to be related to the dependency of the target cell for extracellular asparagine. Most normal tissues contain a measurable level of AS activity that is induced during asparagine depletion by ASNase. Presumably,

this increased AS level provides the cell with sufficient asparagine to maintain protein synthesis during asparagine starvation. Some tumor cells of lymphatic origin, however, express extremely low levels of AS, and therefore are sensitive to the drug. Although there is a presumed lack of AS expression in certain tumors that render the cell sensitive to ASNase, the exact mechanism of cell death on a molecular level is not understood.

ASNase treatment results in a rapid decrease in protein synthesis of affected cells as documented in the 6C3HED mouse lymphoma (Ellem et al., 1970). The depression of protein synthesis is followed temporally by a reduction in the rates of DNA, rRNA and tRNA synthesis. When protein synthesis is blocked with cycloheximide, a similar sequence of inhibition occurs, indicating that the disruption of nucleic acid synthesis is secondary to the reduction of protein synthesis. Although protein synthesis inhibition is the most striking aspect of ASNase treated cells, it is possible that other secondary effects may be responsible for actual cell death. Story et al. (1993) documented that a canine lymphoma treated with ASNase for 4 hours had a 40% fragmentation of chromosomal DNA, consistent with the commencement of apoptosis, with only a 50% depression in protein synthesis. Considering that cycloheximide treatment of lymphocytes was less cytotoxic than exposure to the topoisomerase II inhibitor VP-16 and γ-ray exposure, both of which block cell cycle progression, the authors concluded that other factors, including a disruption in the cell cycle, were likely mediating the rapid onset of apoptosis.

The potential apoptotic effect of a cell cycle blockade in lymphoblasts as a result of ASNase treatment is intriguing, considering the role of asparagine in the cell cycle. Basilico et al. (1987) originally isolated the AS complementary DNA (cDNA) as the gene that relieved a cell cycle block in a Syrian Baby Hamster Kidney (BHK) cell line with a

temperature sensitive mutation (*ts11*) that synchronously arrested the cells at the G1 cell checkpoint when grown at nonpermissive temperature. Repletion of the cells held at the nonpermissive temperature with asparagine resulted in a release of the cell cycle block, identifying this amino acid as the agent responsible for cell cycle control (Greco et al., 1989). Clearly, lymphoblasts that cannot maintain adequate levels of asparagine synthesis are susceptible to not only a depression of protein synthesis, but also a blockade in the cell cycle that may result in apoptosis.

In addition to the effect of ASNase on protein synthesis and the cell cycle, exposure to the drug also may result in the pertubation of metabolic pathways essential to cell growth. As well as the obvious reduction of intracellular asparagine, Ryan et al. (1970) observed that ASNase treatment resulted in decreased glycine levels in sensitive 6C3HED tumors, which potentially could have a negative effect on purine biosynthesis. This ASNase-induced glycine reduction in sensitive tissues was found to be cell-type specific. However, since Keefer et al. (1985) determined that glyoxylate transamination to glycine was actually depressed in the L5178Y lymphosarcoma after ASNase treatment, resulting in the accumulation of potentially toxic levels of the reactive aldehyde, glyoxylate. The decrease in glyoxylate transamination presumably was caused by decreased intracellular asparagine flux through asparagine-glyoxylate aminotransferase.

In addition to changes in glycine metabolism, intracellular glutamine and glutamate levels also are affected by ASNase treatment, which also may affect cell growth and viability. Bussolati et al. (1995) documented that NIH3T3 cells exposed to ASNase undergo apoptosis concurrent with decreases in intracellular glutamine and glutamate. The rapid depression in intracellular glutamine was ascribed to the

glutaminase side reaction of glutamine, which resulted in the exodus and hydrolysis of glutamine. The reduction in the intracellular glutamate level was presumed to be a result of enhanced flux via glutamine synthetase (GS) for the synthesis of additional glutamine, with little to no anionic amino acid transport present to restore intracellular pools of the amino acid. Interestingly, Broome (1968) documented a 12-fold increase in the intracellular level of aspartic acid after ASNase treatment of the 6C3HED lymphoma, implying that extracellular transport of anionic amino acids may be induced as a result of ASNase exposure. Clearly, it appears that there are significant cell-type differences in the susceptibility of certain tumors to ASNase, and that anionic amino acid transport, or amino acid transport in general, may be an important factor influencing cell sensitivity to the drug. The effect of ASNase on glutamine metabolism in human leukemia cells has not been reported previously.

In addition to the intended oncolytic effect on the malignancy, ASNase promotes widespread secondary physiological effects due to systemic amino acid deprivation. Some of the toxic side effects of the drug include gastrointestinal system distress including hypoproteinaemia-related fluid retention, an impairment of CNS function, and renal failure. Some of the effects of protein synthesis blockade also evident in ASNase treated patients include a decrease in production of blood clotting factors leading to thrombic events, decreased serum insulin, and hypoalbuminemia (Lord, et al., 1972; Oettgen et al., 1970; Whitecar et al., 1969). Also, acute pancreatitis is a common complication, occurring in 15% of treated patients (Clavell et al., 1986). Interestingly, the pancreas contains the highest AS protein levels of all rat tissues tested (Hongo et al.,

1992). However, the relationship between this high AS expression and clinical pancreatitis in ALL patients is unknown.

The development of hypersensitivity to ASNase is another potentially life-threatening complication of ASNase administration. Patients given ASNase ultimately develop antibodies to the drug, which may attenuate its pharmacologic effectiveness (Woo et al., 1998). Fortunately, individuals who generate severe allergic reactions to *E. coli* ASNase can be switched to the less immunogenic ASNase from *Erwinia chrysanthemi* with no significant impact on the duration of leukemia-free survival (Larson et al., 1998).

Although the immediate physiological complications of ASNase can be quite severe, the biggest drawback of this therapy is the emergence of drug resistance in relapsed patients. Perhaps the most common phenomenon associated with cellular resistance to ASNase is an enhancement of the expression of AS in the relapsed cell. First recognized in 1968 by Broome using extracts isolated from 6C3HED tumors, ASNase resistant cells had a high rate of AS expression compared to sensitive tumors. Also, an interesting observation that the intracellular concentration of asparagine in sensitive and resistant tumors was similar indicated that ASNase resistant cells efficiently used intracellular asparagine for protein synthesis, and raised the possibility of "channeling" newly formed asparagine toward protein synthesis. Taken together, these data indicate that ASNase resistant cells have an increased rate of synthesis and preferential metabolism for asparagine (Broome, 1968). The phenomenon of increased AS expression in ASNase resistant cells also has been observed in LY1579 cells (Martin et al., 1993), HT 1080 fibroblasts (Andrulis et al., 1990), Chinese hamster ovary (CHO)

cells (Andrulis et al., 1991), U937 lymphocytes (Kiryama et al., 1989), and MOLT-4 lymphocytes (Hutson et al., 1997). Typically, these cells are generated *in vitro* by continual selection in increasing concentrations of the drug. However, studies of lymphoblasts from relapsed patients indicate that the up-regulation of AS occurs *in vivo* as well. Haskell and Canellos (1969) identified 5 ALL patients who were resistant to ASNase, and had a correspondingly higher level of AS activity than 4 patients who were treated successfully with the drug.

The precise mechanism of long-term AS up-regulation in human lymphocytes as a result of ASNase treatment currently is unknown. However, some *in vitro* studies indicated that AS is up-regulated by demethylation of regulatory sequences within the gene. In an asparagine auxotrophic CHO mutant cell line that proliferated in the absence of asparagine, the 5' region of the AS gene was demethylated completely, which corresponded with a high constitutive level of AS expression (Andrulis and Barrett 1989). Also, selection of CHO cells in ASNase resulted in extensive demethylation of the 5' and 3' regions of the AS gene (Andrulis et al., 1991). Interestingly, ASNase caused a 25% reduction in global methylation of the cells, indicating that ASNase affects gene expression by catalyzing the demethylation of AS, and serves as a selective agent for the resistant phenotype.

Although some progress has been made in understanding the long-term expression of AS in ASNase-resistant cell lines, it would also be useful to understand the short-term response of genes potentially involved in asparagine and glutamine transport and biosynthesis in ASNase treated lymphocytes. The amino acid regulation of the AS gene has been the subject of considerable investigation as a model for nutrient control in

mammalian cells, so much of this work can be applied in the context of ASNase-treated cells.

The earliest report of the regulation of mammalian AS by its product was documented by Arfin et al. (1977). After transferring a CHO cell line to asparagine-free medium, the AS enzymatic activity was increased approximately 2-fold. This activity could be returned to control levels by repletion of the medium with asparagine. Also, the heightened AS activity after starvation was determined to be a result of decreased tRNA charging, since culture of a CHO cell line containing a temperature-sensitive mutant $tRNA^{Asn}$ at the nonpermissive temperature resulted in a 3-fold increase in AS enzymatic activity, with no change in AS activity in wild type cells. Interestingly, Andrulis et al. (1979) reported that the culture of CHO cell lines with temperature-sensitive leucyl-methionyl- and lysl-tRNA synthetase mutants at the nonpermissive temperature also resulted in an increase of AS activity, even though the asparaginyl-tRNA remained fully aminoacylated. Also, the blockade of $tRNA^{His}$ charging in FAO hepatoma cells with 5mM of the amino acid alcohol histidinol resulted in a significant induction of AS mRNA (Hutson and Kilberg, 1994), indicating a broad spectrum of control.

The specificity of the amino acid dependent control of AS also has been documented by culture of FAO hepatoma cells in amino acid-free medium and in medium lacking single amino acids. The AS mRNA was fully induced after complete amino acid deprivation for 9 h, and this transcriptional response was blocked by either actinomycin D or cycloheximide, indicating that the synthesis of an "activating protein" was required for up-regulation of the gene (Hutson and Kilberg, 1994). The addition of a single amino acid to the medium partially repressed the AS mRNA induction. However,

the effectiveness of each amino acid varied considerably. Glutamine was the amino acid most effective at maintaining the repression of AS, even at a concentration of 50 μM, approximately one-tenth that of plasma glutamine. Asparagine, histidine, leucine, and free ammonia also were effective repressors, whereas aspartate, glycine, and glutamate were among the weakest amino acids in maintaining the repression of AS mRNA. In converse to the repression of AS in amino acid-free medium by individual amino acids, the removal of histidine, threonine, and tryptophan from otherwise complete medium also resulted in the induction of AS mRNA. Taken together, these data indicate a general promiscuity in the regulation of AS by amino acids, resembling the "general control" response described for amino acid control in yeast. Considering that several amino-acid-degrading enzymes in addition to ASNase have been promoted as antitumor agents, including histidinol (Warrington et al., 1992), threonine deaminase (Wellner et al., 1979), and tryptophan hydroxylase (Schmer and Roberts, 1979), the impact of these enzymes on the induction of AS and the potential implications for ASNase resistance may be significant.

As discussed above, *E. coli* ASNase contains a glutaminase side reaction that results in the depletion and hydrolysis of extracellular and intracellular glutamine. In patients receiving ASNase, plasma glutamine levels fall to below detectable levels during treatment, indicating that this side reaction is a significant occurrence *in vivo* (Miller at al., 1969). This glutaminase action is thought to enhance the anti-neoplastic activity of the drug. Although some clinical data indicate that excessive glutamine depletion is immunosuppressive, no specific studies have been done to elucidate the contribution of glutamine deprivation to lymphoblast cell death. However, some data indicate that in

addition to AS, enhanced GS activity in ASNase resistant cells may contribute to ASNase resistance. Kiryama et al. (1989) documented that U937 cells selected in the presence of ASNase had a 30% increase in GS activity as compared to cells grown in the absence of asparagine. Also, ASNase resistant cells were able to proliferate 50% faster in glutamine-free medium than cells selected in the absence of asparagine, indicating that ASNase had selected for an enhanced ability to grow in glutamine-depleted medium. Although no studies have documented the mRNA expression or the short-term response of GS activity to ASNase in parental and ASNase resistant cells, clearly, the induction of this enzyme may be critical in maintaining of ASNase resistance.

Although intracellular glutamine production may be important in terms of ASNase resistance, the transport of extracellular amino acids that serve as metabolic fuels and macromolecular precursors also must be considered as a factor in drug resistance. The most significant amino acid-regulated transport activity associated with transformed cells is termed System A. This transport agency mediates the Na^+-dependent uptake of small aliphatic amino acids and has been identified in every nucleated cell type tested. In addition, System A is enhanced in transformed cells (Boerner and Saier, 1985; Handlogden and Kilberg, 1988), and correlates with cell cycle progression and cellular growth (Saier et al., 1988; Oxender et al., 1977).

The regulation of System A during ASNase treatment also may be significant in terms of drug resistance and tumor growth, because the activity of this transporter varies inversely with the extracellular concentration of substrate amino acids (Gazzola et al., 1972; Dall'Asta et al., 1978). The first stage of amino-acid-dependent control of System A, which immediately follows removal of substrate amino acids from the culture

medium, is a release from *trans*-inhibition. This phenomenon is thought to occur as a result of the accumulation of intracellular amino acids that "locks" the carrier in a cytoplasmic orientation. During starvation, the exodus of substrate amino acids from the cell releases the carrier from this "*trans*-inhibited" state, resulting in a 2-fold increase in System A activity after amino acid withdrawal (Fong et al., 1990).

The second stage of System A adaptive regulation occurs 1 to 2 h after amino acid starvation, and is characterized by a steady increase in transport activity for a period of 12 to 18 h (Gazzola et al., 1981). The induction of System A is blocked by inhibitors of protein synthesis (Fong et al., 1989), RNA synthesis (Gazzola et al., 1981), poly(A+) adenylation (Kilberg, 1986), and glycoprotein synthesis (Kilberg et al., 1985), indicating that the enhanced activity results from the synthesis of a System A gene product. In addition, the recovery of System A activity in plasma membrane vesicles suggests that System A is a cell-surface glycoprotein (Fong et al., 1990). Recently, the transcriptional induction of System A after histidine starvation of FAO hepatoma cells was demonstrated using Northern analysis (Bain and Kilberg, unpublished results).

During re-feeding of starved cells, the enhanced System A activity undergoes an amino-acid-dependent down-regulation. The first component of down-regulation is *trans*-inhibition, which is due to the accumulation of carriers in the cytoplasmic orientation as the intracellular concentration of substrate amino acids rises (Fong et al., 1990; White and Christensen 1983). The second component of down-regulation is an actinomycin D and cycloheximide-sensitive process that involves the synthesis of a putative short-lived transport inactivating protein (TIP) (Kilberg, 1986; Gazzola et al., 1981; Fong et al., 1990; Kilberg et al., 1985). Interestingly, cordycepin, an inhibitor of

poly(A) polymerase has no effect on transport inactivation, leading to the suggestion that the TIP mRNA may lack a poly(A)$^+$ tail (Kilberg, 1986). Although the lack of a poly(A)+ sequence is exhibited only by histone mRNAs, the relationship between this class of proteins and TIP is unclear.

Currently, the expression of System A, or the other putative neutral amino acid transporters that could potentially mediate the uptake of AS substrates, including Systems ASC, L, and X$^-$A,G have not been characterized in ASNase-resistant lymphocytes. Considering the inter-relationship between amino acid control of AS expression and substrate delivery via plasma membrane transport, the availability of amino acids may be an important process in the maintenance of ASNase resistance.

To gain a deeper understanding of ASNase resistance at the cellular level, the regulation of specific genes by amino acid deprivation must be understood, including those involved in the cell cycle, cellular transport, and amino acid biosynthesis which, in turn, may influence the susceptibility of the cell to ASNase treatment. The goal of this research, therefore, is to determine the expression of amino-acid-regulated genes in the context of ASNase resistance in human lymphocytes. To focus on the mechanisms that may directly influence the delivery of AS substrates and production of intracellular asparagine, this series of experiments examines the regulation of amino acid transporters, AS, and GS at all potential levels of control during ASNase treatment. In addition, the influence of each gene on cellular resistance to ASNase is examined, as are the potential signaling mechanisms that regulate the induction of AS mRNA in response to amino acid and glucose deprivation.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture

The human acute lymphoblastic leukemia cell line MOLT-4 (ATCC CRL 1582) was propagated in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mL/L ABAM (100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B) (GIBCO, Gaithersburg, MD) and 30 μg/mL gentamycin (Sigma, St. Louis, MO). All suspension cultures were maintained at 37°C in a 5% CO_2 incubator (Nuaire, Plymouth MN). Twenty-four hours before all experiments, cells were collected by centrifugation for 5 min at 228 x g, rinsed twice with phosphate buffered saline (PBS) (0.15 M sodium chloride, 10 mM sodium phosphate, pH 7.4), and resuspended at a density of approximately 5 X 10^5 cells/mL in fresh medium.

To establish a model of ASNase resistance, a MOLT-4 subline was created by sequential incubation of parental MOLT-4 cells in increasing concentrations of ASNase (MERCK, West Point, PA) from 1 x 10^{-5} to 1.0 U/mL (Hutson et al., 1997). Cells from this selection process were subcloned by limiting dilution and maintained in RPMI 1640 medium supplemented with 1.0 U/mL ASNase. To examine the reversibility of ASNase effects, resistant MOLT-4 cells were transferred and maintained in RPMI-1640 medium lacking ASNase for a period of six weeks.

Mouse myeloma FDC-P1 cells (kindly provided by Dr. Stratford May, University of Florida Cancer Center) were grown in RPMI-1640 medium supplemented with 10% FBS and 10% WIF-B supernatant containing IL-3, as described (Deng et al., 1998).

FDC-P1 cells stably transfected with a dominant negative c-*jun* N-terminal kinase (JNK) signaling mutant were grown in medium containing 500 μg/mL G418 (Gibco). All other aspects of cell culture and experimental procedures were identical to those of the MOLT-4 cells.

The retroviral HEK293 (human embryonic kidney) "Phoenix-e" cell line (Nolan Laboratory, Stanford University) was propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mM L-glutamine. This Phoenix cell line is stably transfected with ecotropic Moloney Leukemia Virus (MoLV) g*ag-pol* and *env* genes. Phoenix cells were selected for two weeks in 1 μg/mL diptheria toxin and 500 μg/mL Hygromycin B every two months to maintain high levels of the transgenes.

Amino Acid Deprivation

For experiments requiring amino acid starvation, MOLT-4 cells were collected by centrifugation, washed twice with PBS, then resuspended to 4 x 10^5 cells/mL in RPMI-1640 medium deficient in the appropriate amino acid(s). This amino acid-deficient medium was prepared with an RPMI-1640 Select-Amine kit (GIBCO) supplemented with 10% dialyzed FBS and the identical concentrations of antibiotics as the complete RPMI-1640 medium described above. Where applicable, cultures containing ASNase were treated with 1 U/mL of the drug.

ASNase Preparation

ELSPAR ASNase (MERCK), Escherichia Coli type EC-2, was obtained from pharmacy stores, University of Florida. Each vial of ELSPAR contains 10,000 International Units (IU) of ASNase with a specific activity of 225 I U per mg, resulting in

a total enzyme weight of 44.4 mg. In addition, 80 mg of mannitol, an inactive component of the lyophilized preparation is included, bringing the total weight to 124.4 mg/vial. For an active enzyme preparation of 0.5 U/μL, 20 mL of sterile 50% (v/v) glycerol were added to each vial, mixed gently, and stored in 1 mL aliquots at -20°C.

Amino Acid Transport

To determine the amino acid transport rates of MOLT-4 cells, a transport assay adapted to suspension cultures was used. Cells grown for 12 h under various amino acid deprivation conditions were collected by centrifugation, washed twice in choline Kreb's ringer phosphate (KRP) buffer (119 mM choline chloride, 5.9 mM potassium chloride, 1.2 mM magnesium sulfate, 1.2 mM potassium bicarbonate, 5.6 mM glucose, 0.5 mM calcium chloride, 25 mM choline bicarbonate, 0.15% (w/v) bovine serum albumin (BSA)), then incubated for 20 min at 37°C in 40 mL choline KRP buffer to deplete intracellular stores of amino acids. After incubation, the cells were collected by centrifugation, resuspended to 5 x 10^7 cells/mL in choline KRP buffer, and distributed in 96-well plates at 20 μL/well. To initiate transport, 180 μL of a 1.1 X solution containing either glutamine (5.5 μM), asparagine (5.5 μM), or leucine (5.5 μM) with 3.6 μCi [^{3}H]glutamine, 0.18 μCi [^{14}C]asparagine, or 3.6 μCi [^{3}H]leucine, respectively, either in the presence (sodium KRP) (119 mM sodium chloride, 5.9 mM potassium chloride, 1.2 mM magnesium sulfate, 1.2 mM potassium bicarbonate, 5.6 mM glucose, 0.5 mM calcium chloride, 25 mM choline bicarbonate, 0.15% (w/v) BSA) or absence (choline KRP) of sodium, were added to the cells. After incubation at 37°C for 1 min, the cells were harvested by aspiration (15 to 17 mm Hg) with a cell harvester (Brandel, Gaithersburg, MD) adapted to aspirate all of the wells simultaneously, and then deposited on Whatman GF/B filters that had been pre-soaked with 5 mM of the appropriate

unlabeled amino acid to reduce nonspecific binding. After aspiration, the cells were rinsed four times with ice-cold choline KRP. The filters were dried overnight, placed in vials with 3.5 mL of Scintisafe scintillation fluid (Fisher, Pittsburgh, PA), and the radioactivity was determined in a Beckman LS 3810 scintillation counter (Beckman Coulter, Fullerton, CA). To determine the protein content of the MOLT-4 cell transport suspensions, 80 μL of 2% (w/v) sodium dodecyl sulfate (SDS)/0.2 N sodium hydroxide were added to six 20-μL aliquots of each cell suspension, including cell-free choline KRP buffer samples to subtract for the included BSA. Each sample then was subjected to Lowry analysis and was averaged to obtain a final protein value. In an independent experiment, it was determined that there was no significant difference in the protein content per cell of parental, resistant and resistant (-ASNase) cells.

To compute the transport rates of MOLT-4 cells, the specific activity (disintegrations per minute (DPM)/nmole) of the uptake buffers was determined by establishing the DPM in two-20 μL aliquots of each uptake buffer, and then using the following formula: DPM/nmole = [{(average buffer counts per minute (CPM)-background CPM) * (1/isotope counting efficiency)/20μl sample)}/(μM amino acid/1000)]. The DPM of each transport sample was obtained by subtracting the background CPM from experimental samples and dividing by the isotope efficiency according to the following formula: (CPM sample - CPM background)/isotope counting efficiency. Background counts were obtained by harvesting and rinsing buffer samples under identical assay conditions in the absence of cells, and were indistinguishable from scintillation-fluid-only samples. Dividing the resulting DPM by the previously determined protein values (DPM/mg), and dividing this value by the calculated specific

activity of the appropriate buffer (DPM/nmole) * 1000, resulted in a final calculated velocity with units of pmole·mg protein^{-1}·min^{-1}.

ASNase Sensitivity Assay

The sensitivity of MOLT-4 cells to ASNase was determined by a 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) cell proliferation assay (Boehringer Mannheim, Indianapolis, IN). This assay is similar to the 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay commonly used for cell proliferation studies. However WST-1 has the advantages of greater sensitivity and water solubility. Briefly, MOLT-4 cells were collected by centrifugation, were washed twice with PBS, and were resuspended at a concentration of 4 X 10^5 cells/mL in RPMI 1640 medium. The cell suspensions were treated as described in each figure legend, then seeded in 96-well plates at a final volume of 100 µl/well. After incubation for 48 h at 37°C, 10 µL/well of WST-1 reagent were added and the cells further incubated for 2 h under identical conditions. The optical density of the solution, which is linearly related to viable cell number, was read on a plate reader (SLT Lab Instruments) at 450 nm with a reference wavelength of 690 nm. These data are expressed as the percent of control of the mean optical density of the treated wells/mean optical density of the untreated wells ± the standard deviation (SD) of assays performed in quadruplicate.

Apoptosis Assay

The apoptotic profile of cells under various culture conditions was determined by flow cytometry using an Annexin V/fluorescein isothiocyanate (FITC) Apoptosis Detection kit (R&D Systems, Minneapolis, MN). During early stages of apoptosis,

phospatidylserine is exposed to the outer leaflet of the plasma membrane. The Annexin-V conjugated FITC binds to phosphatidylserine in a Ca^{+}-dependent manner, and is detected with a 530 nm filter in a flow cytometer. Propidium iodide (PI) is a DNA binding dye which cannot traverse the plasma membrane of healthy cells. When the plasma membrane is compromised as a result of cell death, PI enters the cells and the necrotic cell is detected with a 670nm filter. For each experiment, MOLT-4 cells were collected by centrifugation, washed twice with PBS, and resuspended at a concentration of approximately 1 x 10^6 cells/100 µL in 100 µL binding buffer (10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, 150 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 1.8 mM calcium chloride). To each 100 µL sample of cells, 10 µL of 50 µg/mL propidium iodide, and 1 µL of 25 µg/mL FITC-conjugated Annexin V were added, and the cells allowed to incubate in the dark at room temperature for 20 min. Following incubation, the samples were subjected to flow cytometry analysis on a Becton Dickinson FACScan instrument. (University of Florida Flow Cytometry Laboratory (FCCL))

For analysis of cells transduced with the viral AS-IRES-GFP constructs, green fluorescent protein (GFP) positive cells were sorted with a FACSort (Becton Dickinson, Franklin Lakes, NJ) instrument with detection filters set at 530 nm. Since the viral infection results in a stable insertion of the transduced gene, the cells were returned to culture medium after sorting and incubated as necessary to obtain the needed number of cells for RNA and apoptosis analysis.

The cell viability analysis of the cells transduced with the AS-IRES-GFP constructs was performed with an assay kit (Pharmingen, Franklin Lakes, NJ) modified to analyze apoptosis and necrosis without background interference from the expressed GFP.

Briefly, MOLT-4 cells were collected by centrifugation, washed twice with PBS, and resuspended at a concentration of approximately 1 x 10^6 cells/100 μL in 100 μL binding buffer. To each 100 μL sample of cells, 5 μL of 7-AAD, and 5 μL of phycoerythrin (PE)-conjugated Annexin V were added, and the cells allowed to incubate in the dark at room temperature for 20 min. The Annexin-V PE was detected at 585nm, and the 7-AAD detected with a long-pass 650mn filter on a Becton Dickinson FACScan instrument. (University of Florida Flow Cytometry Laboratory (FCCL))

Isolation of RNA

Total cellular RNA was isolated with a QIAGEN RNeasy kit (QIAGEN, Valencia, CA). MOLT-4 cells were seeded in 100 mL dishes at a concentration of 4 X 10^5 cells/mL in a total volume of 6 mL. This seeding density and volume was found to be essential for high quality RNA. To harvest the cells, the cultures were decanted into 15 mL sterile plastic tubes, centrifuged at 228 x g for 5 min, and the supernatant removed completely by decanting the tube and blotting upside-down on paper towels. The tubes were quickly vortexed, and 600 μL of QIAGEN buffer RLT were added to the tube while vortexing until the cell pellet was completely dissolved. The cell lysates were homogenized by centrifugation for 2 min at 21,000 x g through a QIAGEN QIAShredder column. RNA was precipitated by the addition of 600 μl 70% (v/v) ethanol, and the resulting precipitates loaded onto an RNeasy column. The columns were washed with subsequent applications of buffer RW1 and RPE according to the manufacturer's directions. Purified RNA was recovered by eluting the column with two separate applications of 50 and 30 μL of RNAase-free water. To quantify the RNA, two separate 1:26 dilutions of each sample were measured at 260 nm in a DU-64 spectrophotometer

(Beckman Instruments, Inc., Fullerton, CA). Yields for this procedure averaged approximately 25 μg RNA/prep with an A_{260}/A_{280} ratio of greater than 1.8.

Northern Analysis

For Northern analysis, 15 μg of total RNA was lyophilized in a Speed-vac concentrator (Savant Corp, Farmingdale, NY) and resuspended in 30 μL of resuspension buffer (12.5 M formamide, 6.6% (v/v) formaldehyde, 6 mM sodium acetate pH 7.4, 0.5 mM ethylenediamine-tetraacetic acid (EDTA) pH 8.0, 20 mM 3-(N-morpholino)propane sulfonic acid (MOPS) pH 7.0). The RNA was denatured by incubation at 65°C for 15 min, placed on ice, and 5 μL loading dye (0.4% xylene cyanol FF, 0.4% bromphenol blue, 1 mM EDTA pH 8.0, 50% glycerol, 0.5 μg/μL ethidium bromide added to each sample. The RNA was size-fractionated through a 1% (w/v) Seakem HGT agarose gel (6.6% formaldehyde, 40 mM MOPS pH 7.0, 10 mM sodium acetate pH 7.4, 1 mM EDTA pH 8.0) overnight at 40 volts. The running buffer (40 mM MOPS pH 7.0, 10 mM sodium acetate pH 7.4, 1 mM EDTA pH 8.0) was constantly recirculated throughout electrophoresis to avoid localized pH gradients. Following electrophoresis, the gel was incubated in 50 mM NaOH for 30 min to depurinate the RNA. After depurination, the gel was soaked in 100 mM Tris pH 7.0 for 30 min to neutralize the base, then soaked in 10X standard sodium citrate (SSC) (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0) for 40 min. The RNA gels were blotted to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) overnight by downward capillary transfer. The RNA was then cross-linked to the membrane by ultraviolet exposure (Setting C-L) in a GS gene-linker UV chamber (BIO-RAD, Hercules, CA) and hybridized to ^{32}P-radiolabeled cDNA probes.

Probe Preparation

The AS cDNA probe was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) from resistant MOLT-4 total RNA using polymerase chain reaction (PCR) specific primers (FWD 5'-TTGTCGACATCACCCTGACCTGCTTACGCC-3' and REV 5'-TTGTCGACGTTCCCCTATCTACCCACAGTCC-3'), based on the published AS cDNA sequence (accession number NM001673). This RT-PCR reaction yielded the 1842 base pair (bp) coding region of the human AS sequence, which was subsequently inserted into the PCR 2.1 vector using a TA cloning system (Invitrogen, Carlsbad, CA). The CHOP/GADD153 cDNA probe was also obtained by RT-PCR from ASNase resistant MOLT-4 cell total RNA. The PCR primers for this sequence (FWD 5'-CTGAGTCATTGCCTTTCTCTTCGGACACTG-3' and REV 5'-GCTCGATTTCCTGCTTGCGCCGTTC-3') resulted in the amplification of a 428 bp fragment of the CHOP/GADD153 mRNA sequence (accession number S40706). The glutamine synthetase (GS) cDNA probe corresponds to exon 1 of mouse kidney GS (accession number J03820). This clone was obtained from American Type Culture Collection (ATCC), and is approximately 87% identical to the human GS cDNA. The ASCT1 probe is a 2102 bp cDNA sequence (accession number L14595), corresponding to the human sodium-dependent neutral amino acid transport activity designated system ASC. The human ribosomal protein L7a probe consists of a 600 bp Pst1 restriction fragment which includes translated and untranslated sequence from the 3' end of the L7a sequence (accession number M36072). This clone was obtained from Dr. Tatsuo Tanaka, University of Ryukyus, Okinawa, Japan. The System A clone is a cDNA probe (accession number 9506836) which corresponds to nucleotides 117-1414 of the coding region.

Plasmid vectors containing the probes of interest were propagated in DH5α *Escherichia Coli* cells, and the DNA prepared by QIAGEN mini-and midi-DNA isolation kits according to the manufacturer's directions. Inserts were isolated from vector backbones by restriction enzyme digestion using flanking restriction sites and subsequent gel purification by size fractionation through 1% Tris-Acetate-EDTA (TAE) agarose gels (0.04 M Tris-acetate, 1 mM EDTA). The bands of interest were visualized by ethidium bromide staining, carefully excised from the gel with a sharp razor blade, and the DNA isolated by a QIAGEN gel extraction kit.

All radiolabeled probes were generated with a Strip-EZ DNA random primed StripAble DNA probe synthesis kit (Ambion, Austin, TX), allowing the membranes to be stripped and re-probed without appreciable loss of signal. Briefly, 25 ng of gel purified cDNA probe were added to a thin-walled PCR tube and incubated in an MJ brand thermocycler (MJ Research, Watertown, Massachusetts) at 100°C for 5 min to denature the DNA strands. The tube was snap-frozen in liquid nitrogen, thawed, and the following components added: 2.5 μL 10X decamer solution, 5.0 μL 5X buffer-dATP/-dCTP, 2.5 μL 10X modified dCTP, 5 μL [α-^{32} P]dATP (3000 Ci/mmol, 10mCi/mL), 1.0 μL exonuclease-free Klenow fragment. The reaction was incubated for at least 30 min at 37°C to ensure maximum incorporation levels. Unincorporated nucleotides were removed by centrifugation through a ProbeQuant G-50 micro spin column (Amersham Pharmacia, Piscataway, NJ) for 2 min at 3,000 x g.

All probes were boiled for 5 min immediately before addition to membranes. Hybridizations were carried out at 61°C in a standard buffer solution (0.5 M Sodium Phosphate pH 7.2, 7% (w/v) SDS, 1 mM EDTA pH 8.0, 1% (w/v) BSA) with the exception of ASCT1, which was hybridized at 42°C in ULTRAhyb buffer (Ambion) to

increase the low abundance signal. After overnight incubation, all membranes except ASCT1 were washed 4 x 10 min with a high stringency wash solution (0.04 M sodium phosphate, pH 7.2, 1 mM EDTA, pH 8.0, 1% (w/w) SDS) at 65°C. The ASCT1 membrane was washed 2 x 5 min with 2X SSC, 0.1% (w/v) SDS and 2 X 15 min in 0.1X SSC, 0.1% (w/v) SDS at 50°C, according to the manufacturer's directions.

The membranes were exposed to BioMax MR film (Kodak, Rochester, NY) and quantified by densitometry using a UN-SCAN-IT software package (Silk Scientific, Orem, UT). Each experiment was repeated at least three times using different batches of cells and medium to ensure reproducibility.

To completely strip the membranes of residual ^{32}P-labeled probe, the membranes were incubated for 10 min at 68°C in probe degradation buffer (Strip-EZ DNA random primed StripAble DNA probe synthesis kit). The probe degradation buffer was removed, and the membranes further incubated at 68°C for 10 min in probe reconstruction buffer. Finally, the membranes were incubated at 68°C for 10 min in 0.1% SDS, and checked with a Geiger counter for residual ^{32}P radioactivity. Typically, this procedure removed all counts after one round of stripping.

Amino Acid Analyses

Parental and ASNase resistant MOLT-4 cells, both cultured for 12 h in the absence or presence of 1 U/mL ASNase, were collected by centrifugation at 4°C, washed twice with 10 mL of ice-cold PBS, and repelleted by centrifugation for 1 minute in a microcentrifuge tube at 10,000 x g. After completely removing the supernatant, the cell pellets were resuspended in 1 mL of 100% ethanol and incubated overnight at –20°C. Following overnight incubation, the samples were centrifuged at 20,800 x g for 30 min at 4°C. A 900 µl sample of each supernatant containing the extracted intracellular amino

acids was collected and dried in a Speed-vac concentrator (Savant, Farmingdale, NY). After dehydration, the pellets were rehydrated with 90 μL ddH_2O and submitted for amino acid analysis by Dr. Kenneth Webb, Protein Nutrition Laboratory, Blacksburg, VA. All samples were run on a reverse phase silica-based free amino acid analysis column (Waters Corporation, Milford, MA), as described {webb reference}. The precipitated cell protein was dried and resuspended in 1 mL of 2%(w/v) SDS/0.2 N sodium hydroxide for Lowry analysis. To determine the amino acid content in the culture medium, 500 μl samples were mixed with 1 mL ethanol and centrifuged at 20,800 x g for 30 min at 4°C. A 1 mL sample of the resulting supernatant was lyophilized and resuspended in 333 μL double-distilled water (ddH_2O) for analysis.

Intracellular Volume Determination

To determine the intracellular water space of MOLT-4 cells, a modification of the technique of Kletzien et al. (1975) was used. Briefly, the uptake of the nonmetabolizable hexose 3-O-methyl glucose (3-MG) was measured by subjecting MOLT-4 cells to a transport assay analogous to that described in "Amino Acid Transport." MOLT-4 cells were collected by centrifugation, washed twice in PBS, resuspended to 5 x 10^7 cells/mL in sodium KRP buffer lacking glucose, then distributed in 96-well plates at 20 μL/well. To initiate transport, 180 μL of a 1.1 X sodium KRP buffer to generate final concentrations of 3-MG (0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 2.5 mM, 5.0 mM) with 6 μL/mL [^{14}C] 3-MG tracer were added to the cells, and the plates incubated for 30 min at 37°C. After incubation, the cells were aspirated (15-17 mm Hg) with a cell harvester (Brandel, Gaithersburg, MD), and deposited on Whatman GF/B filters which had been pre-soaked with 5 mM α-D-glucose to reduce non-specific binding. The cells were

washed four times with ice-cold PBS containing 1 mM phloretin to retain the accumulated 3-MG. The subsequent processing of the filters and Lowry analysis on the original cell suspension were identical to that described under "Amino Acid Transport."

To calculate intracellular water space, moles of 3-MG uptake per milligram of protein was plotted as a function of substrate concentration (mmoles/L), and the slope of the resulting line expressed in terms of microliters per milligram of protein. The specific activity of the buffers was calculated exactly as in "Amino Acid Transport." To determine the background radioactivity, duplicate wells of buffer only were processed exactly as the cell-containing samples.

Immunoblotting

Parental and resistant MOLT-4 cells cultured for 12 h under various amino acid deprivation conditions were collected by centrifugation, washed twice with ice-cold PBS, repelleted, and resuspended in sample dilution buffer (SDB) (0.125 M Tris pH 6.8, 1% (w/v) SDS, 20% (v/v) glycerol, 30 mg/mL bromphenol blue, 715 mM 2-mercaptoethanol). The protein extracts were transferred to microfuge tubes, then incubated overnight at 4°C with end-over-end rotation. Following incubation, the extracts were sonicated by 3 x 5 sec bursts (setting #2), on a Model 60 Sonic Dismembrator (Fisher, Pittsburgh, PA), then centrifuged at 20,800 x g for 30 min at 4°C to pellet insoluble debris. Protein concentration was determined on a 20 μL sample of each supernatant by adding 1.5 mL ice-cold 10% TCA and incubating overnight at 4°C. After centrifugation at 20,800 x g for 30 min at 4°C, the supernatant was carefully removed, and the protein pellet quantified by a modification of the Lowry assay. A 40 μg sample of each extract was diluted 1:1 with SDB, heated at 65°C for 10 min, then subjected to SDS-polyacrylamide electrophoresis through a 7.5% gel. The proteins were

blotted to a nitrocellulose membrane (Protran, Schliecher & Schuell, Keene, NH) by electrotransfer in transfer buffer (25mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS). In order to visualize the blotted proteins, the membranes were immersed in fast green stain (0.1% (w/v) fast green diluted in 50% (v/v) methanol, 10% (v/v) glacial acetic acid) for 5 min, then rinsed in destain solution (50% (v/v) methanol, 10% (v/v) glacial acetic acid) until no background staining was apparent.

To immunoblot AS protein, the membranes were blocked by immersion in blocking buffer (TBS/Tween) (30 mM Tris, 0.1% Tween-20, 200 mM sodium chloride) + 5% (w/v) Carnation non-fat dried milk) for 2h, then incubated in blocking buffer plus AS monoclonal antibody (anti-human AS hybridoma supernatant, University of Florida Hybridoma Facility) for an additional 2 h. The membranes were rinsed 5 x 5 min with wash buffer (TBS/Tween + 1% Carnation non-fat dried milk), then incubated in wash solution plus a 1:20,000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase (K&K, Minneapolis, MN) for 45 min. The membranes were then further rinsed 7 x 5 min with wash buffer, incubated with SuperSignal ECL detection reagent for 1 min (Pierce), then exposed to BioMax MR film to visualize the AS bands.

Glutamine Synthetase Assay

The isolation of cytosolic and membrane fractions for the determination of glutamine synthetase (GS) and glutaminase activity, respectively, was performed by the method of Smith et al. (1984). Parental and resistant MOLT-4 cells cultured for 12 h in the absence or presence of 1 U/mL ASNase, were collected by centrifugation at 4°C, washed twice with ice-cold PBS, and resuspended in 1 mL of a solution containing 0.25 M sucrose, 0.2 mM EDTA, and 2 mM 2-mercaptoethanol (SEB). The resulting cell suspensions were sonicated on ice by 8 X 5 sec bursts (setting #2, on a Model 60 Sonic

Dismembrator) with a cooling interval of 1 min after 4 bursts. Complete cell lysis was verified by microscopic inspection of the sonicated samples. After cell disruption, the lysates were centrifuged at 30,000 x g for 1 hr, the supernatants removed, quantified by Lowry analysis, and stored at -80°C. The pellets were resuspended in 250 μL of SEB by passing 10 X through a 25 ga needle, quantified by Lowry analysis, and stored at -80°C. In an independent experiment, it was determined that there was no loss of GS or GLNase activity following one cycle of freezing and thawing the cell extracts.

Glutamine synthetase activity was measured by a modification of the radioactive ion exchange assay described by Smith et al. (1984). To initiate the assay, 50 μL of cell extract (200 μg protein) were added to 200 μL of a 1.25 X buffer, resulting in a final assay condition of 2.5 mM glutamic acid, 0.25 μCi L-[^{14}C(U)] glutamic acid, 30 mM magnesium chloride, 25 mM ammonium chloride, 10 mM imidizole-HCl pH 7.0, 7.5 mM ATP, 10 mM creatine phosphate (CP), and 1.5 U creatine phosphokinase (CPK). The extracts were incubated for 60 min at 37^{0}C, and the assay stopped by the addition of 1 mL ice-cold distilled water. The 60 min time period used in this assay was taken from Smith et al. (1984), who demonstrated that the assay was linear to 120 min under the conditions described. A 1 mL sample of the resulting suspension was immediately placed on a 2 mL bed volume AG 1-X8 anion exchange "poly-prep" column (200-400 mesh, 8% crosslinked, chloride form, equilibrated in distilled water) (Bio Rad) to bind the remaining glutamate, and rinsed with 6 mL of distilled water. The entire effluent containing the glutamine formed was collected in a 20 mL scintillation vial, to which 10.5 mL of ScintiSafe scintillation fluid (Fisher) were added, and the radioactivity determined in a Beckman LS 3810 scintillation counter. The samples were assayed in triplicate, and the background values obtained from cell extracts incubated in buffer

lacking ATP, CP, CPK, and magnesium chloride were subtracted from samples containing complete buffer to obtain ATP-dependent GS activity. Independent experiments verified that; under the assay conditions described, only 0.3% of radiolabeled glutamic acid came through the column, extracts incubated in buffer lacking ATP, CP, CPK, and magnesium chloride were not significantly different from buffer containing no cell extract, 6 mL of water are sufficient to completely rinse the column of radiolabeled glutamine, and there is no significant quenching effect of radioactive determination due to the added water.

Signal Transduction Pathway Analysis

All the stock solutions for the inhibitors of signal transduction pathways were prepared by dilution in dimethyl sulfoxide (DMSO) to the following stock concentrations: PD98059 (Calbiochem, San Diego, California), 50 mM; SB203580 (Calbiochem), 10 mM; Rapamycin (Calbiochem), 100 ng/μl; Gö6976 (Calbiochem) 50 mM; and Bryostatin (BIOMOL) 20 μM. Rapamycin, Gö6976, and Bryostatin were further diluted 1:10, 1:500, and 1:2 to give final stock concentrations of 10 ng/μl, 0.1 mM, and 0.1 mM, respectively. A 1:1000 dilution of these stock solutions into the appropriate culture medium resulted in the following working concentration of inhibitor: PD98059, 50 μM; SB203580, 10 μM; Rapamycin, 10ng/mL; Gö6976, 10 nM; Bryostatin, 10 nM. To treat the cells with these inhibitors, 120 x 10^6 MOLT-4 cells were harvested, washed twice with PBS, and resuspended in 300 mL of complete RPMI 1640 medium (10% dialyzed FBS) to a concentration of 1 x 10^4 cells/mL. The cells were split into six-50 mL aliquots, and the appropriate inhibitor added at a 1:1000 dilution of the stock solution for a period of 1 hr to pre-treat the cells before nutrient deprivation. Following the 1 hr incubation, each sample of cells treated with a specific inhibitor was

split into four-12 mL aliquots, washed twice with PBS, and resuspended in complete RPMI 1640 (10% dialyzed FBS) medium, or RPMI 1640 medium (10% dialyzed FBS) lacking histidine or glucose. For concurrent treatment with ASNase, 1 U/mL of the drug was added to the appropriate samples in RPMI 1640 (10% dialyzed FBS) medium. Each of the cell populations was seeded in 100 mL dishes, and further treated for a period of 12 h with signaling inhibitors at the identical concentrations as the 1 hr pre-treatment. To extract RNA, the procedure as described in "RNA Isolation" was used with two exceptions. The original volumes (600 µl) of RLT lysis buffer and 70% (v/v) ethanol were doubled to 1.2 mL, and two separate QIAShredders and RNeasy columns were used per sample, reflecting the greater input number of cells.

Reverse Transcriptase - Polymerase Chain Reaction

To obtain a full-length cDNA for AS expression, RT-PCR from resistant MOLT-4 cells was performed. For first strand DNA synthesis, 2 µg of total RNA in 11 µL of ddH_2O was added to 1 µL of an oligo (dT)12-18 primer (500 µg/mL) (Gibco), heated to 70°C to denature the DNA, then quick-chilled on ice. After pulse centrifugation (approx 5 sec), 4 µL of 5 X first-strand buffer (250 mM Tris pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 mM DTT, and 1 µL of a 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP, dTTP) were added to the tube. The mixture was incubated at 42°C for 2 min, and then 1 µL (200 U) of Superscript II reverse transcriptase (RT) (Gibco) added. The reaction was incubated at 42°C for 50 min, after which the reverse transcriptase was inactivated by heating at 70°C for 15 min.

Following first-strand synthesis, full-length AS cDNA was obtained by PCR with *Pfu* Turbo DNA polymerase (Stratagene) to generate high-fidelity transcripts. The PCR

reaction consisted of the following components: 81 μL ddH_2O, 10 μL 10 X Pfu DNA polymerase reaction buffer, 1 μL of a 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP, dTTP), 2 μL first-strand RT reaction, 2.5 μL forward primer (100 ng/μL)(5'-TTGTCGACATCACCCTGACCTGCTTACGCC-3'), 2.5 μL reverse primer (100 ng/μl)(5'-TTGTCGACGTTCCCCTATCTACCCACAGTCC-3'), and 1 μL *Pfu* Turbo DNA polymerase (2.5 U/μL). The reaction was cycled in an MJ brand thermocycler with the following parameters: one cycle of 94°C for 3 min to denature the DNA, thirty cycles of 94°C for 1 min, 60°C for 1 min to anneal primers, 72°C for 2 min to allow extension of the polymerase, and 1 cycle of 72°C for 10 min to allow complete extension of the final transcripts. Because *Pfu* DNA polymerase does not add a single 3'-A overhanging nucleotide to the end of each transcript, and the intent was to use the TA cloning vector, pCR2.1 (Invitrogen), overhanging 3'-A nucleotides were created by adding 0.5 μL *Taq* polymerase (5 U/mL) and incubating at 72°C for 10 min. The PCR reaction was purified by a QIAGEN PCR purification kit prior to TA cloning. Briefly, 500 μL of QIAGEN PB buffer were added to the completed PCR reaction and mixed thoroughly. The entire suspension was loaded onto a QIAGEN mini-spin column, and centrifuged at 20,800 x g for 1 min to bind the DNA. The column was washed with 700 μL of buffer PE before elution of the DNA with 100 μL of elution buffer (10 mM Tris pH 8.5). PCR of the CHOP/GADD153 cDNA was similar to the protocol described for AS with the exception of the use of *Taq* DNA polymerase in the original PCR amplification reaction, obviating the need for the additional 3'-A extension. The CHOP/GADD153-specific primers (FWD 5'-CTGAGTCATTGCCTTTCTCTTCGGACACTG-3' and REV 5'-GCTCGATTTCCTGCTTGCGCCGTTC-3') used corresponded to nucleotides 182-610 of the coding region of human CHOP/GADD153.

Cloning of Human AS

The AS cDNA from the *Pfu* RT-PCR product was cloned into the retroviral vector pBMN-IRES-GFP (obtained from the Nolan laboratory, Stanford University, and provided by Dr. Brad Fletcher) using a TOPO TA cloning kit (Invitrogen). This viral vector contains a polylinker for cloning the gene of interest upstream from an internal ribosome entry sequence (IRES) which, in turn, is upstream of an enhanced GFP sequence. When transduced into target cells, the gene of interest is simultaneously expressed along with GFP, allowing the GFP positive cells, which also contain the gene of interest, to be sorted with a FACS sorting apparatus. Briefly, 2 μL of QIAGEN purified PCR product from the RT-PCR reaction were added to 2 μL of sterile water and 1 μl of pCR 2.1 TOPO vector. The reaction was incubated for exactly 5 min at room temperature and used immediately in a transformation reaction. The transformation utilized 50 μl of TOP10 competent cells (Invitrogen), to which 1 μL of 0.5 M 2-mercaptoethanol and 2 μL of the TOPO cloning reaction were added. After a 30 min incubation on ice, the cells were heat-shocked at 42°C for 30 sec, then incubated on ice for 2 min. A 250 μL aliquot of SOC (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose) was added, then the tube incubated with shaking at 37°C for 1 hour to allow the ampicillin resistance gene to express. The transformation reaction was then spread onto Luria Bertani-ampicillin (LB-AMP) agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 15g/L agar, 100 μg/mL ampicillin) containing 40 μL of a 20 mg/mL solution of 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) in dimethylformamide for blue/white screening.

White colonies indicative of an insertion were picked, grown overnight in 3 mL LB-AMP liquid cultures, and purified plasmid DNA screened for correct orientation by restriction analysis. To clone the AS fragment into pBMN-IRES-GFP without any upstream ATG start sequences that are present in the polylinker of pCR2.1, clones within the pCR2.1 TOPO vector with the AS cDNA orientation on the top strand (opposite and in reverse orientation to that of the T7 promoter) were identified by restriction analysis with Bam HI, which has a single internal restriction site at 885 bp in the AS sequence and a single restriction site in the pCR 2.1 TOPO polylinker. A positive clone was grown for large scale plasmid preparation and verification of the DNA sequence by sequencing (ICBR, University of Florida). To subclone the AS sequence into the Bam HI/Not I pBMN-IRES-GFP polylinker upstream of the IRES sequence, the purified (pCR2.1-AS TOPO) plasmid was completely digested with Not I, and partially digested with Bam HI to release the insert, and the DNA purified by size-fractionation through a 1% (w/v) agarose TAE gel and recovered with a QIAGEN gel extraction kit. To generate compatible ends, pBMN-IRES-GFP was digested with Bam H1 and Not I, then gel purified as described for the AS insert.

The ligation reaction was assembled by combining 50 ng of purified pBMN-IRES-GFP vector cut with Bam HI and Not I, 50 ng of the purified AS insert, 1 X T4 DNA ligase ligation buffer (50 mM Tris pH 7.6, 1 mM magnesium chloride, 1 mM ATP, 5 mM DTT, 5% (w/v) polyethylene glycol-8000) and 1 U (1U/μL) T4 DNA ligase. The reaction was incubated overnight at 16°C in an MJ brand PTC-100 Thermocycler, and 2 μl of the completed reaction used to transform DH5α *Escherichia Coli* cells as described for TOPO TA cloning.

To verify the correct orientation of the AS sequence in pBMN-IRES-GFP, 5 colonies were selected and grown in 3 mL overnight cultures. DNA was isolated using a QIAGEN mini-prep kit, and the purified plasmid vectors subjected to restriction analysis with Bam HI, which has a single internal restriction site at 885 bp in the AS sequence and a single restriction site in the pBMN-IRES-GFP polylinker. A positive clone with the correct AS orientation was grown for large scale plasmid preparation, and an aliquot of the bacterial stock diluted 1:1 in 100% glycerol and placed in the laboratory's clone storage.

A truncated, antisense AS sequence in pBMN-IRES-GFP was also created for knockdown studies. The pCR 2.1 TOPO vector containing AS was digested to completion with the restriction enzyme ApoI. This enzyme is an isoschizmer of EcoRI, which has a restriction site at 443 bp in the AS cDNA. The result is the release of the AS cDNA from the EcoRI/ApoI sites in the polylinker of the pCR 2.1 TOPO vector, as well as truncation of the AS cDNA at the internal EcoRI/ApoI cut site. This 367 bp fragment was gel purified, and ligated to a pCR 2.1 TOPO vector which had been digested with EcoRI, dephosphorylated, and gel purified. The resulting construct is a 357 bp truncated AS with the Bam HI and Not I flanking sites intact for subcloning into pBMN-IRES-GFP. The ligation reaction was set up as described for AS/pBMN-IRES-GFP, and 5 colonies from the subsequent transformation and bacterial plating were screened for the correct orientation (anti-sense) of the truncated AS. To avoid generation of ATG start sequences on the top strand of pBMN-IRES-GFP from the pCR 2.1 polylinker when the AS sequence in the antisense orientation was inserted, it was necessary to verify that the construct was in the same orientation as the T7 promoter (bottom strand). To screen for orientation in pCR 2.1 TOPO, digestion with the restriction enzyme Hind III was

performed. Asparagine synthetase has a Hind III restriction site 71 bp internal to the start of the RT-PCR cDNA. Also, the polylinker of pCR 2.1 contains a single Hind III restriction site upstream of the TA cloning site, allowing the "correct" orientation to be confirmed by the presence of a 286 bp band. This sequence is in the opposite orientation of the previous AS/pCR2.1 TOPO construct, which will generate an antisense transcript when cloned into pBMN-IRES-GFP.

To clone the truncated AS sequence into pBMN-IRES-GFP, the pCR 2.1 TOPO/truncated AS/antisense vector was digested to completion with Bam HI and Not I, and the cDNA gel purified with a QIAGEN gel purification kit. The insert was ligated into pBMN-IRES-GFP that had been previously digested with Bam HI/Not I, and gel purified. The resulting ligation was transformed into bacteria, plated on LB-AMP plates, and 10 colonies picked for screening. As in the previous screening, the pBMN-IRES-GFP/truncated AS/antisense construct was verified by digestion with Hind III. pBMN-IRES-GFP contains a Hind III restriction site directly downstream of the multiple cloning site, which, if the truncated AS was in the "correct" antisense orientation, will release a 71 bp fragment upon digestion. A positive clone with the correct truncated AS orientation was grown for large scale plasmid preparation, and an aliquot of the bacterial stock diluted 1:1 in 100% glycerol and submitted to clone storage.

Plasmid Preparation

All plasmid DNA was prepared with QIAGEN Mini- or Midi- DNA isolation kits, according to the manufacturer's instructions. For the mini plasmid isolations, 1.5 mL of an overnight bacterial culture was pelleted by centrifugation at 20,800 x g for 1 min and the supernatant removed by aspiration. After removal of the supernatant, 250 μL of buffer P1 (50 mM tris-HCl, pH 8.0, 10 mM EDTA, 100 μg/mL RNase) were added, and

the pellet completely resuspended by repeated vortexing. After resuspension, 250 µL of buffer P2 (200 mM NaOH, 1% SDS) were added to lyse the bacteria, and 350 µL buffer PN (3.0 M potassium acetate, pH 5.5) added to neutralize the lysis reaction. The tubes were centrifuged at 20,800 x g for 10 min to pellet cellular precipitates, and the cleared lysate added to QIAGEN mini-columns for binding of the plasmid DNA to the silica-gel matrix. The columns were rinsed by sequential washes of buffers PB and ethanol-containing buffer PE before elution with 100 µL elution buffer (10 mM Tris pH 8.5).

For large scale plasmid DNA preparation, a QIAGEN Midi DNA isolation kit was used in a similar manner as the Mini DNA isolation. To grow the bacteria, 25 mL of LB-AMP was inoculated with 250 µl of a starter culture and allowed to incubate overnight at 37°C with vigorous shaking. The bacteria were pelleted in a 30 mL polyethylene round bottom tube by centrifugation at 6,000 x g for 15 min at 4°C, resuspended by repeated pipetting with 4 mL of buffer P1. The bacteria was lysed with 4 mL of buffer P2 and allowed to incubate at room temperature for 5 min. The reaction was neutralized by the addition of 4 mL buffer PN, and the lysate allowed to precipitate on ice for 20 min before centrifugation at 20,000 x g for 30 min at 4°C. The resulting supernatant was carefully decanted into a QIAGEN-tip 100 column that had been pre-equilibrated with 4 mL of equilibration buffer QBT (750 mM sodium chloride, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol, 0.15% Triton X-100) and allowed to drip by gravity flow. The column was rinsed twice with 10 mL wash buffer (1 mM sodium chloride, 50 mM MOPS, pH 7.0, 15% (v/v) isopropanol, 0.15%), and the DNA recovered with 5 mL of elution buffer (1.25 M sodium chloride, 50 mM Tris-HCl pH 8.5, 15% (v/v) isopropanol). The DNA was precipitated with 3.5 mL of isopropanol, centrifuged at 15,000 x g for 30 min at 4°C, and the pellet washed with 2 mL of 70% (v/v) ethanol. The precipitate was recovered by

centrifugation at 15,000 x g for 10 min at 4°C. To eliminate residual ethanol, the tube was placed in a speed-vac concentrator, and vacuum applied until all traces of ethanol were evaporated. The purified plasmid DNA was resuspended in 500 μL TE and quantified by measuring the absorbance of a 1:25 dilution at 260 nm in a DU-64 spectrophotometer.

Retroviral Infection and Selection

To express AS or the truncated antisense AS in MOLT-4 cells, a moloney leukemia virus (MoLV) retroviral infection system was used. To generate retrovirus, the 293T HEK "phoenix" cell line stably transfected with the *gag-pol* and *env* viral proteins (http://www.stanford.edu/group/nolan/phoenix_info.htmL) with an ecotropic host range (phoenix-E) was transiently transfected with pBMN-IRES-GFP containing the AS constructs or vector alone. The pBMN-IRES-GFP vector contains 3' and 5' retroviral LTR sequences, as well as the ψ packaging sequence, which, when transfected into phoenix cells, generates competent retrovirus that is used to infect sensitive and resistant MOLT-4 cells.

To transfect Phoenix 293HEK cells, 2.5 x 10^6 cells per 60 mm plate in 4 mL DMEM medium were plated 24 h before transfection, which resulted in approximately 80% confluence at the time of transfection. The transfection mixture consisted of 4 μg of plasmid DNA complexed to 12 μL of FUGENE transfection reagent (Boehringer Mannheim, Indianapolis, IN). Briefly, 12 μl of FUGENE were added to 88 μL of serum-free medium and allowed to incubate at RT for 5 min. After the incubation, the mixture was added dropwise to the DNA, and further allowed to incubate for 15 min at RT. After complex formation, the FUGENE/DNA mixture was added to the cells. Twenty-four

hours prior to harvesting the viral supernatant, the medium was replaced with fresh RPMI-1640 +10% FBS and the cells placed at 32°C to maintain stability of the virus.

The viral supernatant was harvested 72 h post transfection by gently collecting the supernatant, and centrifuging at 500 x g for 5 min at 4°C to remove extraneous cells. To infect MOLT-4 lymphocytes, 5 x 10^5 cells stably expressing the ecotropic receptor were pelleted and resuspended in 1 mL of the appropriate viral supernatant that also contained 1 µl of 5mg/mL polybrene. The cells were then spun for 90 min at 500 x g to enhance the infectivity of the viral preparation. After incubation at 32°C for 24 h, the viral supernatant was removed, and the cells were resuspended in fresh medium and cultured at 37°C. The expression of the transgenes was monitored by fluorescence microscopy.

CHAPTER 3
ASPARAGINE SYNTHETASE EXPRESSION AND ASPARAGINASE RESISTANCE

Introduction

The importance of understanding the regulation of genes as a result of amino acid deprivation is clearly apparent when considering the treatment of certain cancers using the strategy of nutrient restriction. Various amino acid degrading enzymes have been identified as antitumor agents, including histidinol (Warrington et al., 1992), glutaminase (Warrell et al., 1980), threonine deaminase (Wellner et al., 1979), methionase (Kreis, 1979), tyrosine phenollyase (Elmer et al., 1979), phenylalanine ammonia-lyase (Abell et al., 1973), and tryptophan hydroxylase (Schmer and Roberts, 1979). The goal of these treatments is to exploit potential metabolic discrepancies between host and malignant cells, which could specifically deprive the tumor of essential amino acids. Unfortunately, there are few differences between the host and tumor in regards to the needs of many amino acids, with the possible exception of asparagine in cells of lymphoid origin. Although asparagine is a non-essential amino acid in terms of the whole animal, it appears that lymphoblastic leukemia cells have a particularly low capacity to synthesize intracellular asparagine via AS, and are therefore dependent on extracellular sources of the amino acid for growth. As a result, the asparagine-degrading enzyme ASNase has become an effective agent in the treatment of acute lymphoblastic leukemia (ALL). However, to appreciate the mechanism of ASNase action and to develop potential

treatments for ASNase resistance, it is essential to understand the effect of amino acid deprivation at the molecular level, particularly upon the AS gene itself.

The ability of individual amino acids to influence gene expression was first recognized in bacterial systems as early as 1961, when Moyed (1961) studied the influence of reduced intracellular histidine on the induction of histidine biosynthetic enzymes. These early experiments indicated that thiazolealanine, a feedback inhibitor of the first enzyme in bacterial histidine synthesis, caused an immediate reduction in growth due to the lowered intracellular levels of histidine, however, because this compound did not interfere with protein synthesis, the biosynthetic operon became de-repressed, and growth continued normally with high activities of compensating enzymes. Subsequent studies by Schlesinger and Magasanik (1964) using the histidine analog α-methylhistidine, which interferes with $tRNA^{His}$ aminoacylation, showed that, in order to exert a regulatory effect, the amino acid under study had to be charged by aminoacylation with its corresponding tRNA. Further evidence of amino acid dependent gene regulation via charged tRNAs was reported by Eidic and Neidhardt (1964), who used a mutant strain of *E. coli* that contained a temperature sensitive mutant of valyl-tRNA synthetase to examine the regulation of the bacterial valine-isoleucine operon. When transferered to a restrictive temperature in valine-containing medium, the valine-controlled enzymes threonine deaminase and acetoacetate synthetase increased in activity 50- to 100-fold in the mutant strain, whereas a leucine-controlled enzyme, α-hydroxy-β-carboxyisocaproate, showed no induction (Eidic and Neidhardt, 1964). Similar experiments using temperature sensitive mutants of $tRNA^{His}$ in *S. typhimurium* also verified that, upon culturing at a nonpermissive temperature, the biosynthetic operon of

histidine synthesis was de-repressed, indicating that the mutant $tRNA^{His}$ was unable to signal the fed state. By increasing the medium concentration of histidine, the amount of histidyl tRNA was correspondingly increased, resulting in repression of the operon (Roth and Ames, 1966).

Extensive regulatory studies of the *trp* and *his* operons in *E. coli* and *S. typhimurium* have shown that two distinct mechanisms control the expression of biosynthetic enzymes in response to amino acid deprivation. The first level of control in the trp operon is analogous to the Jacob and Monod paradigm of negative regulation, and consists of a tryptophan activated repressor protein, *trp*R. The *trp*R regulatory protein is synthesized independently of the *trp* orperon, which consists of a promoter containing an operator binding site, and five downstream structural genes which are transcribed as a polycistronic message, each translated with equal efficiency. In the presence of tryptophan, *trp*R binds to the operator site, limiting the access of RNA polymerase to the promoter (Bennett and Yanofsky, 1978; Oppenheim and Yanofsky, 1980). This mode of control can modulate the expression of the operon approximately 70-fold (Jackson and Yanofsky, 1972), however, it was quickly discovered that mutant *E. coli* strains lacking functional repressor were still competent in regulating the expression of the *trp* mRNA, indicating that repression was not the only method of amino acid dependent control (Jackson and Yanofsky, 1973; Camakaris and Pittard, 1971).

The discovery of deletion mutants of the *trp* operon that had a six-fold increase in the expression of the remaining genes, as well as observations that oligonucleotides corresponding to the first 140 bases of the operon were in concentrations much higher than the complete polycistroinc mRNA, indicated that a process of translation attenuation

also functioned to regulate expression of the *trp* operon (Bronson et al., 1973; Squires et al., 1976). The process consists of initiation of transcription by an RNA polymerase that escapes repressor control, pauses at an upstream transcription pause site, and either continues to complete the transcript or falls off, depending on the position of the lagging ribosome. The attenuation mechanism in the *trp* operon depends on the simultaneous transcription and translation of a 14 amino acid leader peptide, directly upstream of the first structural gene. The position of the ribosome is influenced by the concentration of available $tRNA^{Trp}$, because this leader peptide contains two tandem tryptophan residues. If $tRNA^{Trp}$ is plentiful, the ribosome reads through the peptide, sterically inducing the formation of a termination structure in the RNA. On the other hand, if $tRNA^{Trp}$ is limiting, the ribosome pauses at the tryptophan residues, allowing the formation of a downstream anti-termination structure, allowing transcription to continue (Yanofsky, 1981). Interestingly, the *his*, *phe*, *leu*, and *thr* operons have tandem repeats of the corresponding amino acid contained in the leader peptide, indicating that the mechanism of attenuation via a leader peptide may be applicable to other amino acid controlled operons. Indeed, it appears that attenuation alone is responsible for the regulation of these operons, which explains the regulation of the *his* operon by aminoacylated $tRNA^{His}$ alone. The additional repressive control of the *trp* operon by free tryptophan is postulated to have evolved from the aroH operon, which controls the biosynthesis of aromatic amino acids, because the *trp* repressor also is capable of modulating the aroH operon (Yanofsky, 1981). In terms of AS, the bacterial AsnA gene is activated by the differentially regulated AsnC gene, which codes for a 17-kD protein that has N-terminal

homology to DNA binding proteins catabolite gene activator, *cro*, and *cl* (Kolling and Lother, 1985).

Another potential regulatory mechanism employed by *E. coli* is direct interaction of aminoacylated tRNA to the target enzyme. Duda et al. (1968) reported the binding of $tRNA^{Phe}$ to the first enzyme in aromatic amino acid synthesis in *E. coli*, 3-deoxy-D-arabino-heptulosonate-7-phosphate. The $tRNA^{Phe}$ bound in a charged and uncharged state, however the potential regulatory impact of this association was not reported.

The regulation of genes as a result of nutrient deprivation has been extensively studied in the yeast, *Saccharomyces cerevisiae*. Upon amino acid or purine starvation, the coordinate transcriptional induction of over 40 genes, including 12 different amino acid biosynthetic pathways, is regulated by a "general control" regulatory system, which includes the transcription factor General Control, Non-derepressable 4 (GCN4), a member of the leucine zipper bZIP transactivator family.

The role of cis- and trans-acting factors involved in the nutrient regulation of biosynthetic pathways was first examined by using HIS4-*LacZ* fusion constructs in the context of GCN or General Control, Derepressable (GCD) mutant strains or GCD transfectants. The results of these experiments indicated that although GCN1,2,3 and 5 mutants were found to interact via the GCD1 gene product to affect transcription of biosynthetic genes, GCN4 appeared to have the most direct interaction with the induced genes. In addition, HIS4, which encodes a histidine biosynthetic enzyme, was positively regulated by the GCN response, and that an upstream 5'-TGACTC-3' repeat at position -136 was required for nutrient-dependent control. Interestingly, slight variations in the repeats of this consensus sequence were discovered that were necessary for maximal

transcriptional induction. Taken together, these studies indicated that GCN4 was an activator that directly interacted with amino acid regulated genes. In support of this hypothesis, Hope and Struhl (1985) demonstrated specific binding of the GCN4 gene product to the HIS3 gene where the 5'-TGACTC-3' sequence is repeated 6 times in the promoter region, however, no binding occurred on identical sequences on non-regulated genes. The presence of a downstream a poly dT tract found in responsive promoters is thought to be necessary for transcriptional activation. Using dilutions of purified GCN4 to determine the relative binding affinity to different GCN4 consensus sites, Ardnt and Fink (1986) determined the optimal sequence for GCN4 binding to be 5'-RRTGACTC-3' followed by a stretch of thymidines. These studies indicated that the presence of slightly different consensus sequences may modulate GCN4 binding and transcriptional activation of different genes. Sellers et al. (1990) showed that GCN4 bound two symmetrical half sites with a variable internal base pair (5'-ATGA(C/G)TCAT-3'). Upon mutagenesis of this putative GCN4 recognition sequence, it was discovered that the protein binds these two half sites, but the optimal central base is a cytosine. Additionally, an insertion of two central bases was tolerated by GCN4, indicating that the protein can recognize different spacing motifs, a feature that is not shared with ATF/CREB proteins in spite of identical half-site consensus sequences.

Based on evidence from electromobility shift analysis (EMSA) experiments, it was determined that GCN4 bound DNA as a homodimer, with the DNA binding domain encompassing the C-terminal 60 amino acids (Hope and Struhl, 1987). This DNA binding domain shares significant homology to the mammalian transcription factor, *jun*, which also binds DNA at a similar consensus site (Vogt et al., 1987). The critical amino

acids that comprise the activation domain of GCN4 are centered in a 19 bp acidic region, which can be completely separated from the C-terminal DNA binding domain (Hope and Struhl, 1996). Recently, a mammalian homologue of the *Drosophila* nuclear receptor FTZ-F1, multiprotein bridging factor 1 (MBF1), has been found to interact with GCN4 to mediate transcriptional activation by bridging the DNA binding region of GCN4 and TATA binding protein (TBP) (Takemaru et al., 1998).

The full length GCN4 mRNA transcript is 1534 bp in length, with a distinctive series of four, short open reading frames (ORFs) interspersed in 590 bp of leader sequence 5' to the GCN4 coding region. Hinnebusch (1994), based on a series of mutations of the upstream ORFs, has proposed a model based on ribosome scanning to account for the translational regulation of GCN4 under amino acid deprivation conditions. During amino acid repletion, the 40S ribosome-eukaryotic initiation factor 2 (eIF2)-guanidine triphosphate (GTP)-tRNAi^{Met} initiation complex binds the 5' cap, and scans toward the GCN4 coding region. When this complex reaches the AUG initiation sequence of ORF1, it binds a 60S ribosomal subunit, and the ORF is translated with a concomitant hydrolysis of the bound GTP and release of tRNAi^{Met}. After dissociation of the ribosomes, re-initiation of scanning is accomplished by the rapid binding of a new eIF2-GTP-tRNAi^{Met} ternary complex to the 40S subunit and further translation of one of the remaining ORFs 2-4. After dissociation from ORF 2-4, the ribosomes are unable to re-initiate at the GCN4 start site. Although the exact mechanism of ORF4 as a translational barrier is unknown, it appears that the termination region of this ORF is considerably more effective at preventing downstream re-initiation than ORF1, which actually seems to promote re-initiation. During amino acid starvation, the efficiency of

eIF2-GTP-tRNAi^{Met} association with the 40S ribosome is reduced due to a decrease in the level of ternary complex. Because tRNAi^{Met} is required for AUG recognition, the ribosome continues to scan past ORFs 2-4 after translation of ORF1, and finally initiates at the GCN4 start codon after binding the ternary complex. The response of this system to upstream signaling as a result of amino acid starvation depends, therefore, on a sufficient eIF2-GTP-tRNAi^{Met} concentration to initiate translation, but at a reduced level that decreases the association between the ternary complex and the 40S ribosome, allowing the ribosome to continue scanning past ORF4. Based on studies that followed GCN4 protein levels as a function of induction of target genes, no post-translational mechanism is thought to be involved in the regulation of GCN4, indicating that the scanning translation model is the sole site of amino acid regulation for this transcription factor (Albrecht et al., 1998).

The recycling of GTP on eIF2 regulates the concentration of active eIF2-GTP-tRNAi^{Met} ternary complex available for binding to the 40S ribosome, and thus affects the efficiency of ribosome re-initiation. This guanidine diphosphate (GDP) to GTP exchange is mediated by eIF2B, a complex consisting of the GCD1, GCD6, GCD2, GCD7, and GCD3 proteins. During the amino acid starved state, the concentration of free eIF2B is reduced by sequestration of this complex by enhanced binding onto phosphorylated eIF2. Upon phosphorylation of the α subunit of eIF2, the GCN3, GCD7, and GCD2 subunits of eIF2B bind more tightly to eIF2, preventing the association of eIF2B with other eIF2 complexes. In addition, there is a conformational change in the GCD1 and GCD6 subunits of eIF2B that prevents GDP-GTP exchange on the eIF2 γ subunit, further preventing recycling of eIF2-GDP. Thus, a reduced effective concentration of eIF2B

ultimately results in a decrease of eIF2-GTP-tRNAi^{Met} complex available for association with 40S ribosomes, allowing the scanning mechanism to read through ORF4 of the GCN4 gene and begin translation at the authentic start site (Hinnebusch, 1997).

The amino acid dependent phosphorylation of eIF2 is mediated by the GCN2 gene product. Upon starvation for amino acids, there is no increase in the protein level of GCN2, so the effect is mediated by functional stimulation (Dever et al., 1993). Based on sequence homology, GCN2 shares similarity to the catalytic domain of eukaryotic protein kinases. Also, mutagenesis of a homologous lysine residue believed to be active in the phosphotransfer reaction of eukaryotic protein kinases abolishes GCN2 activity (Wek et al., 1989), indicating that this protein acts as an amino acid-sensitive protein kinase. Interestingly, the C-terminal domain of GCN2 is homologous to yeast and mammalian hystidyl-tRNA synthetases (HisRSs), including a core anti-parallel β-sheet "motif 2" sequence that interacts with the acceptor stem of the tRNA (Ruff et al., 1991). Based on the fact that aminoacyl-tRNA synthetases bind uncharged tRNAs, the HisRS motif of GCN2 has been implicated in the binding of uncharged tRNAs as the mechanism of sensing the level of free amino acids. Indeed, Wek et al., (1995) have demonstrated yeast tRNA binding to the HisRS motif site using northwestern blot analysis. Cells undergoing starvation for amino acids or containing a nonfunctional aminoacyl-tRNA demonstrate increased eIF-2α phosphorylation by GCN2. Also, this region has been shown to be essential for the kinase function if GCN2 *in vivo* and *in vitro*, indicating that the activity of GCN2 is regulated by uncharged tRNA binding to the HisRS motif (Wek et al., 1995).

Recently, two additional yeast proteins, GCN1 and GCN20 have been implicated in GCN2 activation by uncharged tRNAs. Mutant strains of GCN1 and GCN20

demonstrate significantly reduced phosphorylation of eIF-2α, but do not decrease GCN2 kinase activity, indicating that a GCN1/GCN20 complex mediates the activation of GCN2 by uncharged tRNA (Marton et al., 1993, Vazquez de Aldana et al., 1995).

The association between tRNA charging and AS expression in mammalian cells was first examined by Gallo and Longmore (1970), who performed chromatographic analysis on $tRNA^{Asn}$ isolated from ASNase sensitive L5178Y and resistant L1210 murine leukemia cells. This study indicated that L1210 cells highly induced in their expression of AS have a distinct $tRNA^{Asn}$ profile as compared to the sensitive L5178 cells, implying that differences in $tRNA^{Asn}$ charging may be important in the regulation of AS. This hypothesis was confirmed by Arfin et al. (1977), who used a CHO cell line containing a temperature sensitive mutant of asparaginyl-tRNA (Andrulis et al., 1978). When placed at a nonpermissive temperature, these cells exhibited a 2- to 3-fold increase in AS activity which could be reversed by the addition of asparagine to the culture medium. Also, demonstrating the breadth of the AS response, CHO cell lines containing temperature sensitive leucyl- methionyl-, and lysyl- tRNA synthetase mutants underwent AS induction at a nonpermissive temperature, even when the $tRNA^{Asn}$ was fully charged (Andrulis et al., 1979). The dependence of tRNA charging for the regulation of AS was confirmed in rat FAO hepatomas by Hutson et al., (1994), who observed an induction of AS during treatment of the cells with the amino acid alcohol, histidinol. This study also identified seven amino acids capable of inducing AS mRNA expression when removed from the medium. Taken together, these data indicate that AS in mammalian cells is regulated by aminoacylated tRNAs, and is subject to a general amino acid control response similar to the general control model described for yeast.

The study of the control of AS at the molecular level was initiated by the isolation of the mammalian AS gene. To facilitate cloning, Ray et al., (1984) used a CHO cell line that had been induced in AS expression over 300-fold by culturing in increasing concentrations of the glutamine analog albizziin. From these cells, an enriched AS library was created from size-fractionated poly A+ mRNA and probed with ^{32}P-labeled cDNA. The differential hybridization of parental and selected cells permitted the identification of the induced AS cDNA. Using the CHO cDNA as a probe, the human AS sequence was subsequently isolated from a human fibroblast library. When this sequence was transfected into Jensen rat sarcoma cells (JRS), which ordinarily are auxotrophic for asparagine, asparagine independence was conferred to transferrant cells (Andrulis et al., 1987).

AS was also isolated as a human cDNA that was capable of allowing progression through the G1 phase of the cell cycle in a Syrian BHK cell line that had a temperature sensitive mutation (*ts*11), synchronously arresting the cells at this checkpoint when grown at nonpermissive temperature (Basilico et al., 1987). The nature of the mutation was discovered to be a C to T transition, resulting in a leucine to phenylalanine substitution, creating a heat-labile phenotype of the AS protein (Basilico et al., 1990). The functional nature of AS as the gene that relieved the cell cycle block was further confirmed when BHK *ts*11 cells held at nonpermissive temperature were relieved of the cell cycle block upon the addition of exogenous asparagine (Greco et al., 1989).

The genomic structure and intron-exon boundaries of AS were mapped by the two separate groups that originally isolated the AS cDNAs (Andrulis et al., 1989; Zhang et al., 1989; Greco et al., 1989). The complete gene consists of approximately 2 kilobase

(Kb) of coding sequence interspersed within 21 Kb of genomic DNA and contains 13 exons, the first two of which are noncoding (Greco et al., 1989). The preliminary analysis of the sequences required for expression of the AS gene was performed by cloning 5' genomic regions into a chloramphenicol acetyl transferase (CAT) reporter vector and transfecting these constructs into HeLa and NIH 3T3 cells. From deletion studies of an initial 3.4 Kb region containing 280 bp of upstream sequence, exons 1 and 2, and introns 1 and 2, two promoter sites, termed P1 and P2, were identified that had putative regulatory activity. The P1 region was centered approximately 280 bp upstream of the first exon, and contained 2 putative Sp1 binding sites and multiple transcription start sites. The P2 region, although much weaker in potential regulatory capability as P1, was located in the first intron and contained promoter features such as a TATA box, OTF-1 binding sites and a simian virus 40 (SV40) enhancer core (Greco et al., 1989). The ability of the 3.4 Kb fragment to respond to amino acid regulation was shown by stably transfecting the BHK ts11 cell line with a CAT vector/3.4 Kb construct. Upon exposure of the cells to nonpermissive temperature, an increase in CAT mRNA was detected which was prevented by the addition of exogenous asparagine. Furthermore, when transiently transfected into CHO cells, the CAT/3.4 Kb AS construct also responded to leucine starvation, demonstrating that this fragment was capable of inducing a general amino acid response (Guerrini et al., 1991). Further deletion analysis from the 3' and 5' ends of the 3.4 Kb fragment revealed that a DNA sequence from -164 to +44 of the AS promoter was sufficient for basal and amino acid dependent gene expression. To map precisely the DNA region conferring amino acid regulatory properties to the AS promoter, scanning mutagenesis of the -150 to +1 region was then performed in

conjunction with transient CAT assays of the appropriate constructs. The results of these experiments revealed a short region between nucleotides -70 and -64 that was essential in conferring amino acid responsiveness to transfected CAT constructs. Interestingly, the deletion of the SP1 sequences abolished basal activity, but had no effect on amino acid dependent regulation. Also, no mutants exhibited an increase in activity over basal levels, indicating that a positive element was responsible for the observed induction (Guerrini et al., 1993).

To isolate a potential regulatory protein, EMSA experiments were performed using radiolabeled oligonucleotides corresponding to the putuative amino acid response sequence. An oligonucleotide containing a sequence that conferred amino acid-dependent responsiveness to the AS gene generated 3 distinct bands when incubated with HeLa nuclear extracts, whereas an oligonucleotide containing a mutation that abolished amino acid-dependent regulation only generated 2 of the 3 bands. Through additional mutation of the oligonucleotides that generated the additional band, the core sequence determined to be necessary for amino acid-dependent responsiveness was 5'-CATGATG-3'. This sequence was centered on nucleotide -68 and termed the amino acid response element (AARE). Interestingly, EMSA assays using an AARE-containing oligonucleotide incubated with nuclear extracts from starved and fed HeLa cells showed no differences in gel shift patterns or intensity. The authors interpreted these results as evidence that secondary protein interactions may be responsible for amino acid responsiveness (Guerrini et al., 1993).

In terms of the putative P2 promoter located within the first exon, it was determined by Guerrini et al. (1993) that this region had considerable homology to a *fos*

intragenic regulatory element (FIRE) motif contained in the first untranslated exon of amino acid regulated genes including *fos*, *jun*, *myc*, and ornithine decarboxylase (ODC). When coinjected into mouse fibroblasts with the serum response element (SRE), the FIRE sequence induced *fos* expression in quiescent cells, indicating that this sequence was a negative regulatory motif (Lamb et al., 1990). Using EMSA assays, it was determined that a protein bound to the putative FIRE sequence in a single-strand dependent manner, however, this binding was not amino acid-dependent, as there were no changes in the pattern or intensity of band shifts when the oligonucleotides were incubated with nuclear extracts from fed or starved HeLa cells. Targeted mutations of specific residues within the FIRE sequence necessary for protein binding were introduced into AS/CAT constructs and the vectors transfected into HeLa cells. The expression of AS was then monitored in transfected cells that were either fed or amino acid deprived. Although basal expression of AS was induced, indicating the potential disruption of a sequence necessary for binding of a negative regulatory element, amino acid dependent regulation of the construct was unaffected (Guerrini et al., 1993). Collectively, these data demonstrated that the FIRE sequence was not involved in amino acid-dependent regulation, despite the apparent similarity with the *jun* and *fos* promoters.

Although a significant degree of AS expression can be attributed to transcriptional regulation, the induction and down-regulation of AS also appears to have a component of post-transcriptional regulation in some mammalian cells. Gong et al. (1991) showed that the decrease in AS mRNA levels observed after the addition of asparagine to BHK *ts11* cells held at a nonpermissive temperature could be prevented by the RNA transcription inhibitor actinomycin D, as well as the translational inhibitors, cycloheximide and

puromycin, indicating that RNA and protein synthesis were necessary for the down-regulation of AS after refeeding. To demonstrate the post-transcriptional regulation of AS in the absence of potentially confounding inhibitors, an AS cDNA under the control of a constitutive SV40 promoter was introduced into *ts11* cells and stable transfectants isolated. Using primer extension to identify the AS mRNA derived from the SV40 promoter, it was determined that the levels of AS mRNA under SV40 promoter control were increased when the cells were incubated at a nonpermissive temperature, illustrating that trans-acting factors were perhaps involved in the stability of AS mRNA in response to amino acid deprivation (Gong et al., 1991).

A series of mutagenesis experiments by Gong et al. (1991) further explored the relationship between AS translation and mRNA stability. Five separate constructs of AS with in-frame stop codons at positions 23, 60, 204, 398, and 464 were created and placed under the control of the constitutive SV40 promoter. BHK *ts11* cells, were stably transfected with the appropriate vectors and placed at a nonpermissive temperature to induce AS expression. Interestingly, the constructs with stop codons at 23 and 60 failed to induce when the cells were placed at a nonpermissive temperature, however, the 204, 398, and 464 mutants were capable of accumulating mRNA, indicating that translation of AS mRNA was essential for its increased stability during asparagine limitation. When these constructs were placed under the control of the AS promoter, all the constructs were induced upon asparagine depletion, however, the 398 and 464 mutants were significantly more inducible than the 23, 60, and 204 mutants (Gong et al., 1991). Taken together, these data indicate that AS is regulated both transcriptionally by cis-acting elements, and post-transcriptionally by RNA stability and translation-dependent mechanisms, however,

these mechanisms may be relatively cell-specific. The stimulation of AS mRNA in FAO cells following complete amino acid starvation could be completely blocked by the RNA transcriptional inhibitor actinomycin D, as well as the translational inhibitor, cycloheximide. These data were interpreted to indicate that transcription inhibition alone prevented the accumulation of AS mRNA, however the possibility exists that the synthesis of a trans-acting regulatory factor may be necessary for AS mRNA induction (Hutson and Kilberg, 1994). Also, no increase in half-life for the AS mRNA was observed when JRS cells, an asparagine auxotroph with no endogenous AS activity, transfected with AS cDNA were starved for all 20 amino acids, indicating that, at least in the rat sarcoma, no post-translational stabilization of the AS mRNA occurs during amino acid deprivation (Hutson, unpublished data).

Although the short-term regulation of AS has been primarily studied by transient transfections and short-term starvations, long-term *in vitro* studies using various amino acid analogs and metabolic inhibitors have been useful in examining the long-term effects of asparagine limitation on AS regulation, which may be applicable to ASNase selected cell lines and ASNase-resistant human leukemias. The ability of cells to up-regulate AS expression as a means to escape drug selection was demonstrated *in vitro* using CHO cells cultured in the asparaginyl-tRNA and AS inhibitor, β-aspartylhydroxamate. CHO cells that were selected in asparagine-limiting medium in the presence of the aspartate analog had AS activity levels 5-fold greater than that of parental cells. Significantly, these cell lines appeared to have permanently up-regulated AS activity, with no change in the Km or thermal stability of AS (Gantt et al., 1980). Although this suggested that the expression of AS, and not the functional property of the enzyme was altered, upon two-

step mutagenesis of CHO cells with ethyl methane sulfonate (EMS) and subsequent selection in β-aspartylhydroxamate, AS overproducing cells were generated that were enhanced in both their expression of AS and affinity for β-aspartylhydroxamate (Andrulis and Siminovitch. 1982).

The overproduction of AS was also achieved in CHO cells by single-step mutation with EMS and selection with the glutamine analog, albizziin. Long-term culture in increasing concentrations of albizziin resulted in concomitant enhancements of AS activity, up to 260-fold at 50 mM (Andrulis at al. 1985). Karyotypic analysis of these cells indicated chromosomal breaks and translocations on chromosome 1 in cells producing 40-fold or less AS, while homogenously stained regions (HSR) were prominent on chromosome 1 in cells expressing AS at levels above 40-fold induction (Andrulis et al., 1983). These regions of putative gene amplification were not observed in the β-aspartylhydroxamate selected cells, indicating that different mechanisms of AS overexpression may occur, depending on the selective agent. This phenomenon was also observed in human HT 1080 fibrosarcoma cell lines selected in increasing concentrations of albizziin and ASNase. The HT 1080 cells selected in albizziin had extensive gene amplification and AS overexpression, whereas the ASNase selected cells, although induced in AS mRNA and protein, did not display evidence of gene amplification (Andrulis et al., 1990)

The genetic basis for AS overexpression in selected cell lines was further investigated by Sugiyama et al. (1983), who determined that the JRS cell can be stably converted to asparagine independence by treatment with the nucleoside analog, 5-azacytidine, suggesting that DNA methylation was responsible for AS silencing. More

detailed studies were conducted by Andrulis and Barrett (1989) using an asparagine auxotrophic CHO mutant to characterize the methylation status of the AS genomic region. Employing the technique of methyl-sensitive restriction analysis, it was determined that the entire 5' region of the AS gene in the auxotrophic cells was completely methylated. Conversely, spontaneous revertants which were asparagine independent and expressed high levels of AS exhibited extensive demethylation of the 5' region, suggesting a role for DNA methylation in the long-term up-regulation of AS. These studies were subsequently extended to investigate the effect of chemotheraputic drugs, including ASNase, on the methylation of AS (Andrulis et al., 1991). Restriction analysis of the selected cell lines indicated that only ASNase caused a significant reversion to asparagine prototrophy, and that the selected cell lines had extensive demethylation of the 5' and 3' regions of the AS gene. Interestingly, it was also determined that ASNase treatment caused a 25% reduction of 5-methyl-cytosine in the cells. Accordingly, starvation of the cells with asparagine alone caused a 10% drop in methylation, indicating that long-term amino acid limitation may not only cause global demethylation, which may affect short- and long-term gene expression, but also may act as a selective agent for the induced phenotype (Andrulis et al., 1991).

Although these studies are useful in formulating a general model of long-term AS regulation, the cell lines utilized may not be applicable in predicting the response of human lymphocytes to ASNase treatment. These non-lymphoid cells may express certain genes, for example, amino acid transporters, which are not found in lymphoblasts, strongly influencing the potential mechanisms of drug escape. Experiments on AS expression in lymphocytes have been conducted by Kiriyama et al. (1989), who

characterized several lymphocyte cell types on their degree of ASNase resistance. Lymphocytes could be grouped into a "Non-sensitive" group, which included T-lymphoblastiod JM cells, B-lymphoblastoid RPMI-8866 cells, and non-B lymphoblastoid HPB-NULL cells, and a "Sensitive" group including the T-lymphoblastoid MOLT-3 and MOLT-4 cells, as well as the promyelocytic U937 cells. It was determined by culturing the cells in increasing concentrations of ASNase that U937 cells could be rendered resistant to ASNase, with a corresponding 80-fold induction of AS activity. U937 cells could also be made resistant to ASNase, although to a lesser degree, by long-term selection in asparagine-free medium. These asparagine-free selected cells displayed an AS activity 7-fold greater than that of wild type U937 cells. Interestingly, the AS activity of ASNase resistant U937 cells cultured for over 5 months in medium lacking ASNase and containing a 5-fold excess of asparagine remained unchanged for both the ASNase and asparagine-free selected cell lines, indicating that long-term, irreversible modification of AS expression had occurred as result of long-term amino acid deprivation (Kiriyama et al., 1989). In a separate study, Hutson et al. (1997) showed that the T-lymphoblastoid MOLT-4, the non-T-non-B lymphoid NALL-1 and B-lymphoblastoid B-ALL cells had AS mRNA and activity levels inversely proportional to the intrinsic degree of ASNase resistance. Also, MOLT-4 cells selected in increasing concentrations of ASNase had elevated AS activity, mRNA, and protein levels, but displayed no evidence of AS gene amplification, similar to that observed in ASNase resistant CHO cells isolated by Andrulis et al. (1991)

In terms of human leukemias, the clinical data regarding the expression of AS in patient cells resistant to ASNase are scarce, and are often complicated by cross-resistance

from other chemotheraputic agents, however, some limited studies on AS expression in patient samples where ASNase was the sole inductive agent have been conducted. Haskell and Canellos (1969) reported that the AS activity in leukemic cells from patients resistant to ASNase treatment exhibited AS activity levels 7-fold greater than that of sensitive patients. Accordingly, the ability of cells to grow *in vitro* in the absence of asparagine is a useful prognostic marker for the treatment of ALL. In a 1969 study, 18 of 21 patients whose cells were dependent on ASNase to proliferate *in vitro* achieved a complete resistance, whereas only 1 of 9 children whose cells required exogenous asparagine for growth achieved a complete remission. Furthermore, Kaspers et al. (1997) have used *in vitro* "challenge assays" to predict successfully the response of patients to various chemotheraputic drugs, including ASNase, indicating that leukemic cell lines generated by drug selection may serve as useful models for ASNase resistance in human leukemias.

Another potential complicating factor to consider when examining the genetic selection of ASNase resistant cells *in vivo* is the glutaminase side reaction present in the *E. coli* preparation of the drug. Although the activity of the glutaminase reaction is only 2-3% of the ASNase activity, the enzyme is given in sufficient doses as to reduce the plasma levels of glutamine significantly (Miller et al., 1969). Plasma glutamine depletion is thought to be the cause of immunosuppression observed after ASNase administration. Indeed, this was conclusively shown by using an ASNase preparation from the bacterium *Vibrio succinogenes,* which had no glutaminase activity. After administration of the *E. coli* enzyme for 4 days, spleen weights, thymus weights, thymic cell number, and immunoglobulin bearing B-lymphocytes were all reduced in the treated mice, whereas

mice exposed to ASNase produced from *Vibrio succinogenes* were indistinguishable from control (Durden and Distasio, 1980). In addition to immunosuppressive effects, the simultaneous depletion of asparagine and glutamine also may have a bearing on the selection of resistant cells. Kiriyama et al. (1989) observed that U937 cells selected in ASNase could survive in glutamine-free medium, whereas cells selected in asparagine-free medium could not, indicating that growth in ASNase-containing medium as compared to asparagine-free medium selects for additional glutaminase-resistant factors.

Clearly, there is a general lack of information regarding the expression of AS in human lymphocytes as a result of ASNase treatment. The purpose of the experiments in this chapter, therefore, is to examine the short- and long- term regulation of AS in response to ASNase treatment, and to determine whether enhanced AS expression is the sole agent responsible for ASNase resistance in human lymphocytes.

Results

Viability of MOLT-4 cells during ASNase Treatment

The MOLT-4 cell line used in this study was originally isolated from a juvenile ALL patient, and represents an immature lymphoid lineage cell that expresses TdT, and does not express the cell surface antigens CD4 and CD8. Parental MOLT-4 cells are sensitive to ASNase treatment, however, to generate a model of relapsed cells, a resistant MOLT-4 subline has been generated by sequential incubation of the parental cells in increasing concentrations of ASNase (Hutson et al., 1997).

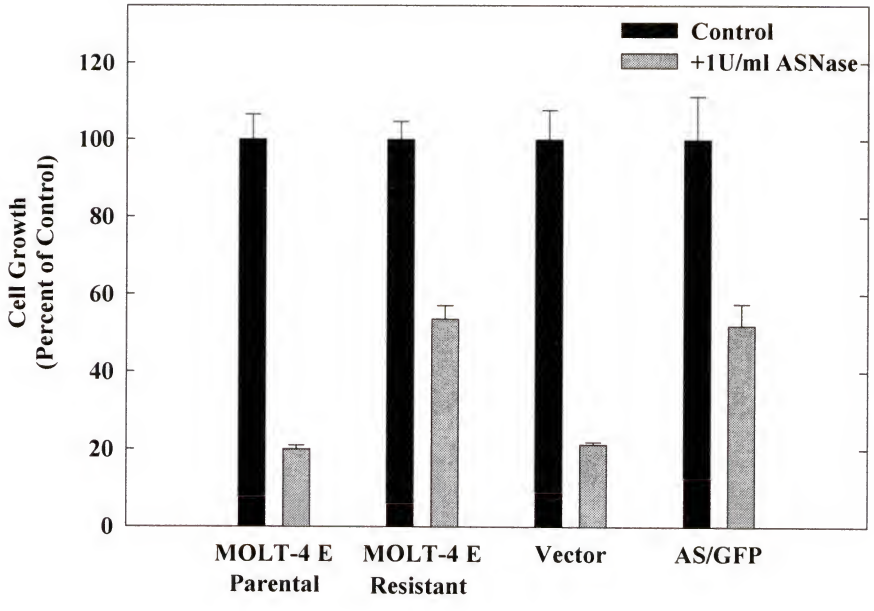
To determine the viability of parental and resistant MOLT-4 lymphocytes in the presence of ASNase, cells were incubated in the absence and presence of 1 U/mL ASNase for 48 h, upon which WST-1 assays and an annexin-V apoptosis assays were

performed. The WST-1 assay, as detailed in Chapter 2, is a colorimetric reaction that measures the activity of the mitochondrial electron transport chain, which can be interpreted as a measure of viable cell number. The annexin-V apoptosis assay measures cell death in terms of two parameters: apoptosis, or programmed cell death, and necrosis. The generation of membrane asymmetry and the consequent exposure of phosphatidylserine to the outer surface of the plasma membrane is an early marker of apoptosis, and is detected by FITC-conjugated annexin-V, which binds phosphatidylserine in a Ca^{2+}-dependent manner. The later stages of apoptosis and eventual necrosis is determined by the ability of the fluorescent dye propidium iodide to enter into compromised cell membranes and bind DNA. Using a combination of these three methods gives a complete picture of the response of MOLT-4 cells to ASNase treatment and the possible correlation with AS expression.

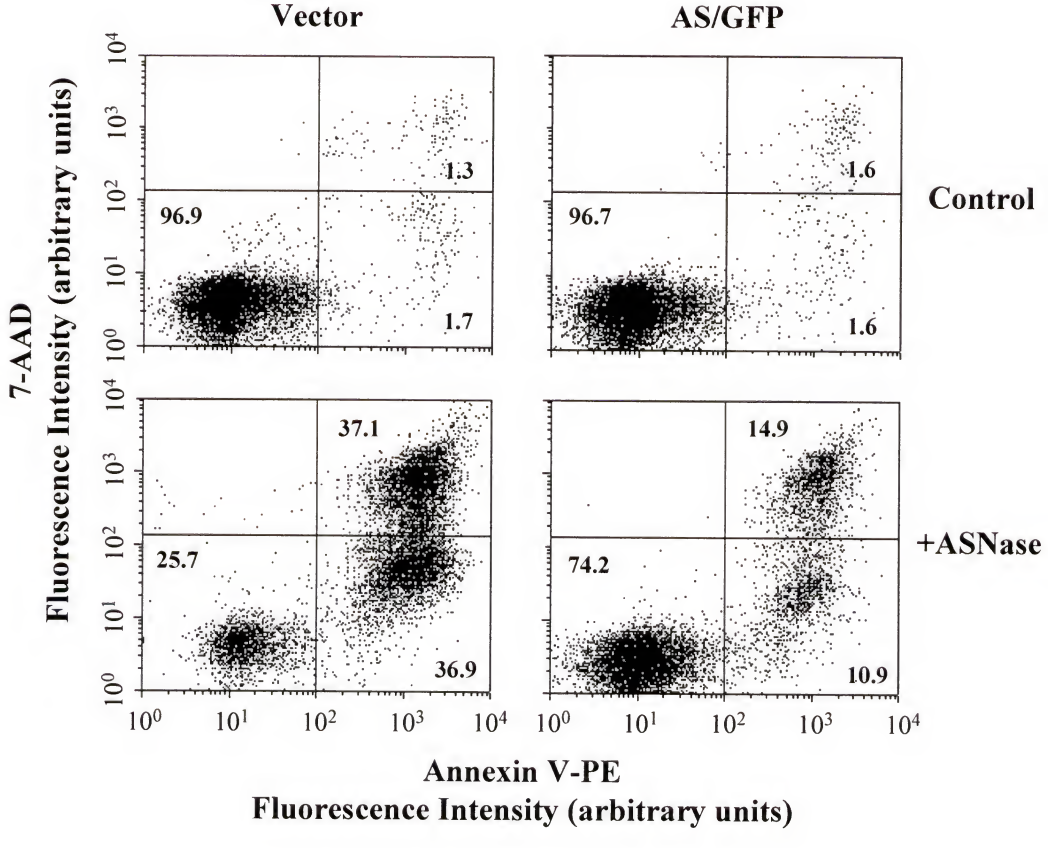
When parental cells were incubated in the presence of ASNase for 48 h, there was a 90% reduction in viable cell number, indicating that ASNase had a significant effect on the proliferation of these cells (Figure 3-1). As indicated by the apoptosis data as in Figure 3-1B, 64% of the cells were undergoing apoptosis or had been killed at this time point. For resistant cells, there was also a reduction in cell number for ASNase-treated cells as compared to untreated controls, however, this value was much less (approximately 20%) than in parental cells. Strikingly, there was no difference in apoptosis between the ASNase-treated and untreated resistant cells, indicating that the reduction in cell number for resistant cells was solely a factor of increased growth rates of untreated cells as compared to ASNase-treated samples. It is unclear from these data,

Figure 3-1. Effect of ASNase on Cell Growth and Viability in MOLT-4 Cells. To determine the effect of ASNase on cell growth and viability, parental and resistant MOLT-4 cells (1×10^4 cells/mL) were incubated for 48 h in the absence (control) or presence (+ASNase) of 1 U/mL ASNase and performing a WST-1 cell proliferation assay (A) and an annexin V-FITC apoptosis assay (B) and as described in Chapter 2. The reversibility of ASNase resistance was determined by incubating resistant cells in the absence of ASNase for 6 weeks (Resistant 6 wk-ASNase) before analysis. For the WST-1 cell growth assay, the absorbance of the treated cells is plotted as a percentage (± SD, n=4) of the untreated control for each individual cell line. For the apoptosis analysis, the numbers in each quadrant indicate the percentage of cells from a total of 10,000 counted. The data presented are representative of three independent experiments.

A


Control
+1U/ml ASNase
Cell Growth
(Percent of Control)
120
100
80
60
40
20
0
MOLT-4 E
Parental
MOLT-4 E
Resistant
Vector
AS/GFP

B


Vector
AS/GFP
Control
+ASNase
7-AAD
Fluorescence Intensity (arbitrary units)
96.9
1.3
1.7
96.7
1.6
1.6
25.7
37.1
36.9
74.2
14.9
10.9
Annexin V-PE
Fluorescence Intensity (arbitrary units)

however, whether a subpopulation of the ASNase treated cells is restricted at a specific cell cycle checkpoint, or if the population as a whole is subject to a permanent or temporary reduction in cell proliferation. To determine the long-term effects of ASNase treatment on MOLT-4 cells, resistant cells normally cultured in the presence of the drug, were cultured in the absence of ASNase for 6 weeks (6 wk -ASNase). The response of resistant cells cultured in the absence of ASNase for 6 weeks shows an intermediate level of cell proliferation between the parental and drug-maintained cell lines that correlates with AS expression (Figure 3-1, 3-2). It is evident by the low amount of cell death as indicated by the annexin-V apoptosis assay, that these 6 wk -ASNase cells have undergone permanent genetic changes as a result of ASNase selection, and more closely resemble the drug-maintained phenotype.

Amino Acid Analysis of Parental and Resistant MOLT-4 Cells

To determine the effect of either short-term (12 h) or long-term ASNase treatment on MOLT-4 cell amino acid content, the extracellular medium and deproteinized total cell extracts of parental and resistant cells were subjected to amino acid analysis. As shown in Table 3-1 (columns 1 and 4), a 12 h incubation in 1 U/mL ASNase caused a complete depletion of culture medium asparagine and a 95% reduction in glutamine with concomitant increases in aspartate and glutamate. Relatively small changes occurred in the extracellular concentrations of all the remaining amino acids, however, a tendency towards lower concentrations of specific amino acids including arginine, asparagine, cysteine, glutamine, glycine, isoleucine, leucine, lysine, and serine in the resistant controls as compared to the parental control cells was observed. Measurement of intracellular amino acids also revealed a decline in parental cell asparagine and glutamine

content in response to a 12 h ASNase treatment (Table 3-2). Resistant cells maintained in ASNase contained no asparagine or glutamine, however, incubating the ASNase resistant cells for only 12 h in the absence of drug resulted in a complete recovery of the intracellular asparagine and glutamine. Interestingly, in spite of the large ASNase-induced increase in extracellular aspartate and glutamate levels (Table 3-1), the intracellular aspartate and glutamate concentrations were actually decreased in ASNase-treated parental or resistant cells (Table 3-2, compare column 1 with column 2 or 4). This result is consistent with the observation to be discussed in Chapter 4, that both cell populations lack significant anionic amino acid transport, thus, even 2 mM extracellular glutamate (Table 3-1, columns 2 and 4) in the medium of ASNase treated cells does not result in an increase in the intracellular glutamate concentration.

Asparagine Synthetase Expression in MOLT-4 Lymphocytes during Amino Acid Deprivation.

To determine the level of AS expression in parental and resistant MOLT-4 lymphocytes as a result of short-term or long-term ASNase treatment, cells were incubated for 12 h in complete RPMI-1640 medium + 10% FBS in the absence and presence of 1 U/mL ASNase. Northern analysis of the isolated mRNA revealed a major band corresponding to approximately 2.0 Kb when probed with the human AS cDNA (Figure 3-2). A photographic negative of the 18S ribosomal RNA has also been shown to confirm the consistent loading of each lane. As expected, parental cells cultured in the absence of ASNase exhibited extremely low AS mRNA levels. This observation is consistent with the susceptibility of these cells to ASNase treatment because of a putative

Table 3-1. Extracellular Amino Acid Levels After ASNase Treatment of MOLT-4 Cells. To determine the extracellular amino acid levels of MOLT-4 cultures after ASNase treatment, parental and resistant cells (1×10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1U/mL ASNase for 12 h and the culture supernatant subjected to amino acid analysis as described in Chapter 2. The data presented are the averages ± standard deviations of samples analyzed in triplicate, and is representative of three independent experiments.

Extracellular Amino Acid Levels of Parental and Resistant MOLT-4 Cells After 12h ASNase Treatment

	Parental Control		Parental +ASNase		Resistant Control		Resistant +ASNase	
Amino Acid	**AVG (μM)**	**SD (μM)**	**AVG (μM)**	**SD (μM)**	**AVG (μM)**	**SD (μM)**	**AVG (μM)**	**SD (μM)**
Alanine	93.5	0.5	113.3	1.9	92.9	6.4	76.8	1.3
Arginine	762.1	9.1	757.6	6.1	592.3	79.9	743.4	14.8
Asparagine	346.9	11.3	0.0	0.0	250.3	43.1	0.0	0.0
Aspartate	129.2	1.1	376.1	13.5	97.8	14.8	403.1	5.2
Cysteine	203.4	12.5	224.9	7.5	160.1	37.0	223.1	2.6
Glutamine	853.7	17.9	18.0	2.6	598.7	92.5	10.5	0.8
Glutamate	196.6	7.0	1121.6	25.6	172.6	26.1	1074.8	12.4
Glycine	139.1	1.3	149.1	3.9	105.3	16.0	142.2	4.3
Histidine	56.4	2.6	62.7	1.4	46.5	3.9	59.3	2.4
Isoleucine	317.8	2.3	312.8	2.5	258.5	19.4	292.0	9.2
Leucine	311.1	0.5	313.9	1.4	238.4	25.9	288.1	7.7
Lysine	151.9	1.6	161.1	1.6	108.1	26.3	147.7	4.9
Methionine	59.9	0.3	62.5	0.3	45.6	2.9	57.6	0.6
Phenylalanine	71.0	0.2	75.7	0.9	57.1	7.7	68.7	1.2
Proline	168.2	2.8	164.8	0.5	156.7	0.4	162.6	3.5
Serine	191.2	5.7	213.9	6.9	138.6	19.3	176.2	7.4
Threonine	132.7	6.8	145.1	1.7	110.3	1.9	131.3	0.1
Tryptophan	18.0	0.3	19.4	0.4	14.5	1.6	17.6	0.4
Tyrosine	86.3	0.3	90.7	1.4	64.5	6.3	83.7	1.3
Valine	155.3	1.2	160.8	2.9	138.8	10.0	148.3	3.7

Table 3-2. Intracellular Amino Acid Levels After ASNase Treatment of MOLT-4 Cells. To determine the intracellular amino acid levels of MOLT-4 cultures after ASNase treatment, parental and resistant cells (1×10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and the collected cells were subjected to amino acid analysis as described in Chapter 2. The data presented are the averages ± standard deviations of samples analyzed in triplicate, and is representative of three independent experiments

Intracellular Amino Acid Levels of Parental and Resistant MOLT-4 Cells After 12h ASNase Treatment

	Parental Control		Parental +ASNase		Resistant Control		Resistant +ASNase	
Amino Acid	AVG (μM)	SD (μM)	AVG (μM)	SD (μM)	AVG (μM)	SD (μM)	AVG (μM)	SD (μM)
Alanine	7.4	0.5	16.3	0.7	11.1	1.1	20.5	0.8
Arginine	1.9	0.3	2.0	0.1	1.5	0.2	1.5	0.2
Asparagine	9.3	0.6	0.0	0.0	9.9	1.0	0.0	0.0
Aspartate	8.6	0.8	6.4	0.3	7.4	0.8	2.3	0.1
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glutamine	27.7	12.4	1.6	0.0	23.3	2.3	2.7	3.0
Glutamate	65.6	6.3	32.4	2.2	65.1	4.6	15.5	0.5
Glycine	15.0	0.9	28.1	2.0	19.5	1.9	38.7	2.7
Histidine	0.0	0.0	0.0	0.0	1.2	0.1	1.6	0.0
Isoleucine	3.0	0.2	4.6	0.2	5.0	0.5	7.2	0.3
Leucine	3.1	0.2	4.9	0.2	5.0	0.5	7.3	0.3
Lysine	0.7	0.0	0.0	0.0	0.5	0.1	0.5	0.0
Methionine	1.4	0.1	1.9	0.1	1.8	0.2	2.3	0.1
Phenylalanine	1.0	0.0	1.6	0.1	1.6	0.2	2.3	0.1
Proline	10.9	0.6	36.2	17.7	59.5	1.7	38.4	0.5
Serine	6.3	0.3	18.3	1.4	6.8	0.7	19.5	1.7
Threonine	8.0	0.3	18.5	1.2	8.7	0.7	18.2	1.1
Tryptophan	0.0	0.0	0.0	0.0	0.5	0.1	0.6	0.0
Tyrosine	0.7	0.1	1.3	0.1	1.5	0.2	2.3	0.1
Valine	1.3	0.1	2.5	0.1	2.4	0.3	3.6	0.1

lack of endogenous asparagine production. Interestingly, parental cells have a high capacity for up-regulation of the AS mRNA when treated with ASNase, even exceeding the AS mRNA levels of the resistant MOLT-4 cells. This result is inconsistent with the common belief that parental cells are sensitive because of an inability to induce their expression of AS in response to ASNase treatment. Resistant cells, however, appear to have a higher basal level of AS mRNA expression, as seen in lane 3 of figure 3-2. This higher basal expression of AS mRNA may be an important factor in the selective advantage of these cells during ASNase treatment, and is consistent with studies by Andrulis et al. (1991) and Kitada et al. (1994) that demonstrated permanently up-regulated AS expression after long-term amino acid deprivation. Resistant cells also were blunted in the degree of AS mRNA up-regulation in response to ASNase treatment, perhaps owing to the higher basal expression of AS, and, by extension, increased intracellular asparagine at the time of ASNase treatment (Figure 3-2, lane 4).

In order to assess the long-term impact of ASNase treatment on AS expression, resistant cells were cultured in complete RPMI-1640 medium +10% FBS in the complete absence of ASNase for 6 weeks (6 wk -ASNase) (Figure 3-2, lane 5). These cells appeared to have basal AS mRNA levels at an intermediate level between the sensitive and resistant cells (Figure 3-3), indicating that, although some basal expression is lost due to long-term culture in the absence of the selective agent, a permanently induced AS mRNA component is present in the resistant cells. In order to clearly see the basal level of AS expression in the (6 wk -ASNase) cell line as compared to parental control cells, a darker exposure of the Northern blot is shown in figure 3-3. The 6 wk -ASNase cells also had induced AS mRNA levels exceeding that of both parental and resistant cells when

each was acutely treated with ASNase for 12 h(Figure 3-2, lane 6). This greatly enhanced AS mRNA in the 6 wk -ASNase induction may arise from two factors: a higher basal level of AS expression than parental cells which could potentially result in a higher capacity for up-regulation, but a lower basal expression than resistant cells maintained in the presence of the drug, resulting in a greater amino acid starvation response than the ASNase-maintained resistant cells.

In terms of protein levels, the low expression of AS in parental cells is clearly evident by a barely discernable band in Figure 3-4 when immunoblot analysis is performed with the AS monoclonal antibody. Also, although the AS mRNA level of parental cells is induced to a high degree during ASNase treatment, exceeding the AS mRNA levels of the resistant cells, the protein level is not increased to the same extent. It is possible that the 12 h incubation is not sufficiently long enough to observe the full extent of the AS protein induction, perhaps, paradoxically, as a result of reduced protein synthesis due to low AS expression. At later time points, however, these cells begin to undergo apoptosis, so the full extent of AS protein expression in sensitive cells may not be realized before cell death.

As compared to parental cells, resistant cells have an extremely high AS protein content in both the fed and ASNase maintained samples, despite the considerable discrepancy of the mRNA levels between the two conditions (Figure 3-4, lanes 2,3). It should be noted that the ASNase treated resistant cells should be considered at a "basal" level, because ASNase is present continuously in the culture medium for these cells. As a result, the decay of the AS mRNA or protein within the experimental 12 h time frame must be considered as a factor in resistant samples, since amino acid-induced genes will

presumably be constitutively expressed at a high level during routine culture in ASNase. Accordingly, the AS mRNA must have a relatively short half-life of 2 h in ASNase-free medium, which probably indicates that after 12 h of growth in complete medium, steady-state mRNA levels are reached, however, the half-life of the AS protein is approximately 20 h, regardless of the extracellular concentration of amino acids. This indicates that in the fed resistant cells, fully repressed levels of AS protein may not be reached within 12 h of refeeding with medium lacking ASNase, exaggerating the non-induced magnitude of AS protein in resistant cells.

Resistant cells that have been cultured in the absence of ASNase for 6 weeks display an intermediate level of AS protein expression in the fed state, consistent with the pattern observed for AS mRNA expression (Figure 3-4, lanes 5,6). In contrast to the resistant cells maintained continuously in the presence of ASNase, however, the basal expression of AS in the 6 wk -ASNase cell line may truly be considered repressed. Consequently, it is clear from these cells that a long-term adaptive increase in constitutive AS protein expression has occurred relative to the parental cells, potentially resulting in enhanced ASNase resistance.

Because ASNase depletes the circulating levels of glutamine as well as asparagine in ALL patients, (Miller et al., 1969) it was of interest to determine whether the signal for AS induction was initiated by the absence of asparagine, glutamine, or a combination of both. Additionally, since ASNase actively depletes asparagine from the medium and acts as a "sink" for this amino acid, the induction of AS under ASNase treatment was compared to starvation for individual amino acids.

Figure 3-2. Expression of Asparagine Synthetase mRNA During ASNase Treatment of MOLT-4 Cells. To assess the transcriptional regulation of AS during ASNase treatment, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and subjected to northern analysis as described in Chapter 2. The reversibility of AS mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis (Resistant 6 wk-ASNase). The blot (15 μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph.

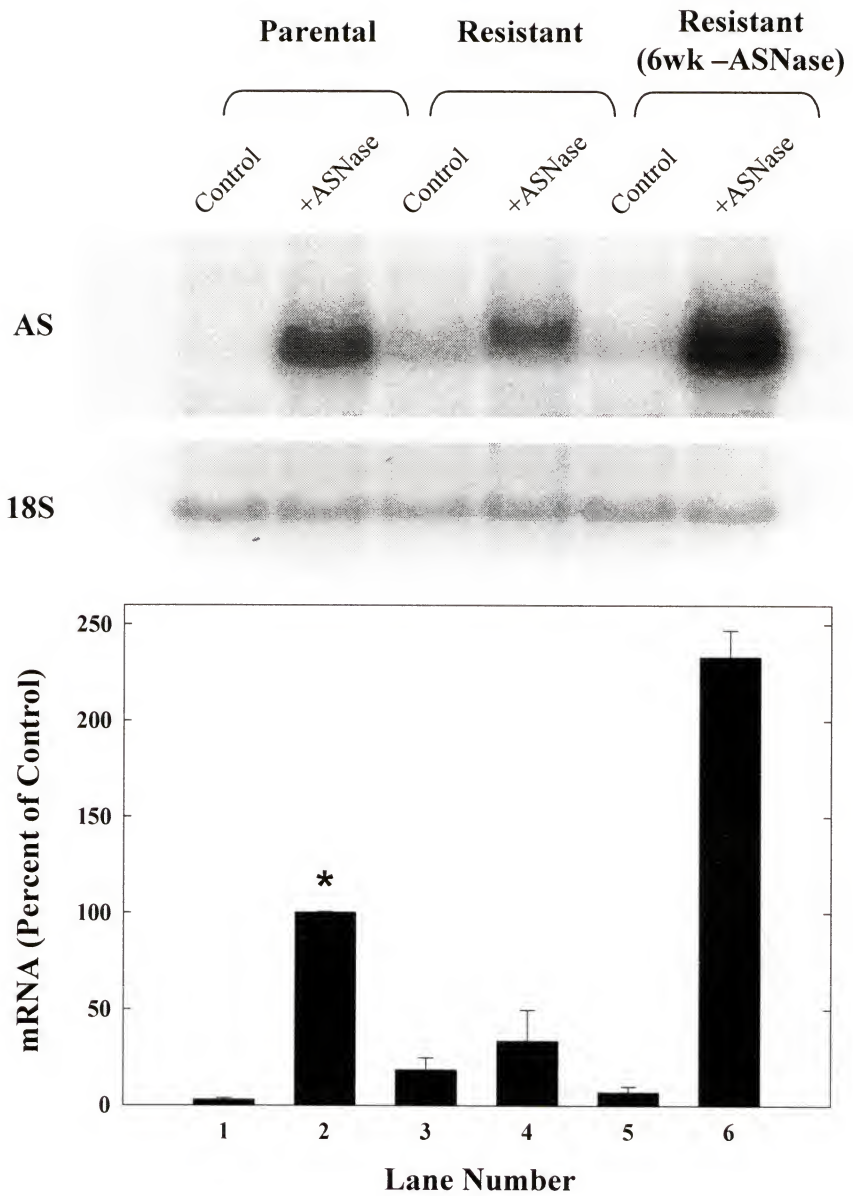

Parental
Resistant
Resistant
(6wk –ASNase)
Control
+ASNase
Control
+ASNase
Control
+ASNase
AS
18S
mRNA (Percent of Control)
250
200
150
100
50
0
*
1
2
3
4
5
6
Lane Number

Figure 3-3. Long-term Expression of Asparagine Synthetase mRNA After ASNase Treatment of MOLT-4 Cells. To assess the long-term transcriptional regulation of AS after ASNase treatment, parental cells cultured in the absence of ASNase and resistant MOLT-4 cells continuously cultured in 1 U/mL ASNase were compared to resistant cells cultured in the absence of ASNase for 6 weeks (6 wk -ASNase), by northern analysis as described in Chapter 2. The blot (15 μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to resistant cells incubated in the presence of ASNase and plotted as a bar graph.

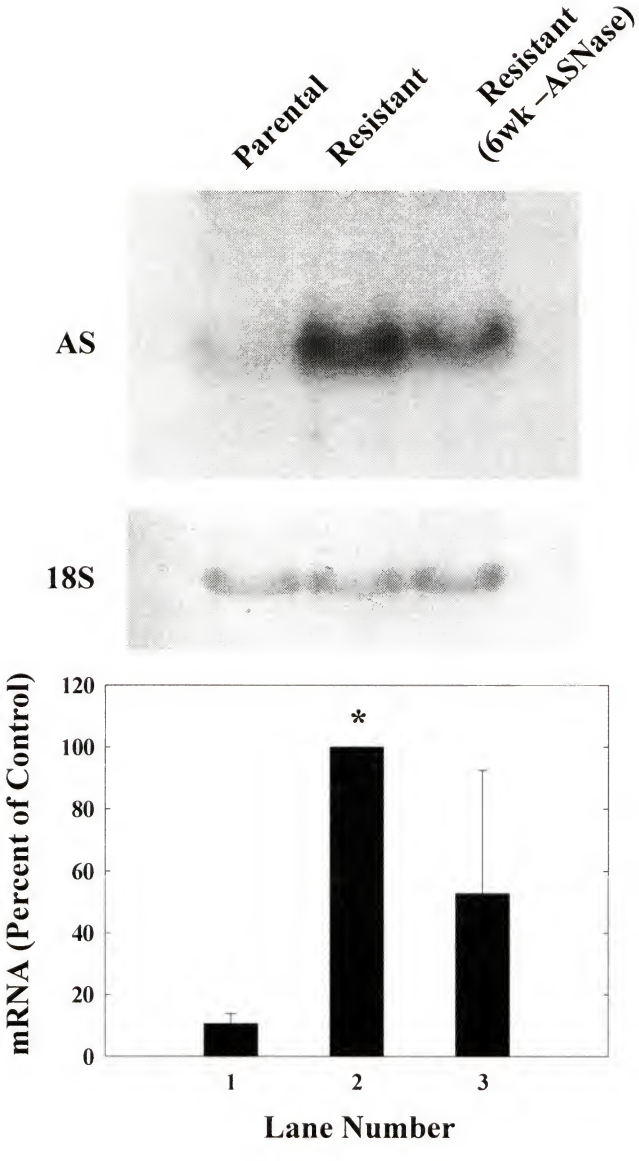
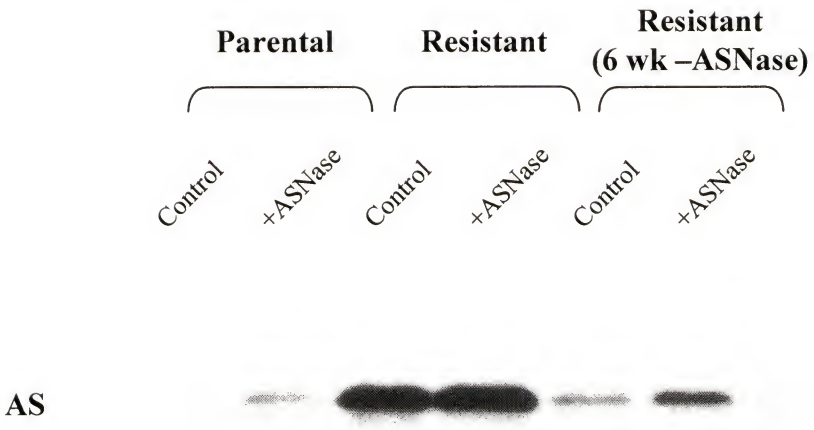
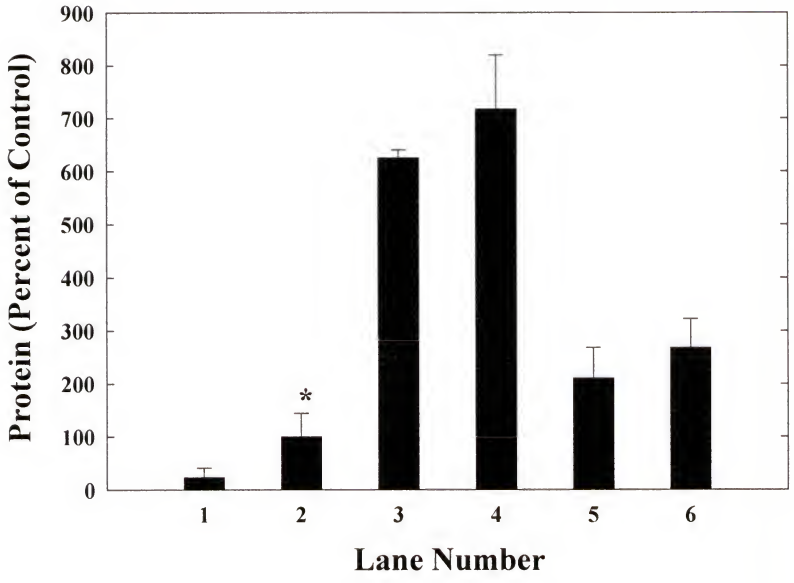

Parental
Resistant
Resistant (6wk –ASNase)
AS
18S
mRNA (Percent of Control)
120
100
80
60
40
20
0
*
1
2
3
Lane Number

Figure 3-4. Expression of Asparagine Synthetase Protein During ASNase Treatment of MOLT-4 Cells. To determine the regulation of AS protein in response to ASNase treatment, parental and resistant MOLT-4 cells (1×10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and subjected to immunoblot analysis as described in Chapter 2. The reversibility of AS mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis (Resistant 6 wk-ASNase). The blot (40 μg/lane) was probed with a monoclonal antibody to AS and visualized with a goat anti-mouse horseradish peroxidase (HRP) secondary antibody (A). The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph (B). The data presented are representative of three independent experiments.


Parental
Resistant
Resistant
(6 wk –ASNase)
Control
+ASNase
Control
+ASNase
Control
+ASNase
AS


Protein (Percent of Control)
900
800
700
600
500
400
300
200
100
0
*
1
2
3
4
5
6
Lane Number

Parental cells, as expected, exhibited a strong ASNase-dependent stimulation of AS mRNA (Figure 3-5). When starving for asparagine alone, the magnitude of this induction was almost as great as ASNase treatment, indicating that depletion of asparagine from the medium is almost as effective as continuous enzymatic degradation. When depleting for glutamine to dissect the effect of the glutaminase side reaction of ASNase, AS was induced to a lesser degree than following asparagine starvation. Starvation for both amino acids together resulted in an increase of AS mRNA which was lower than that of ASNase treated cells. These observations may be the result of enzymatic degradation resulting in a more complete starvation for asparagine, because intracellular free asparagine and asparagine derived from protein turnover that may be exported extracellularly are targets for ASNase, whereas starvation alone represents the withdrawal of the amino acid from the medium at the start of the incubation only. The response of AS expression to the active depletion of asparagine may be marginally higher as a result.

When drug resistant cells were incubated with ASNase or starved for asparagine, there was a 2-fold induction of AS mRNA, which was to be expected if high basal levels of AS are indeed sufficient to prevent, or at least blunt, a starvation response (Figure 3-5, lanes 6,7). Resistant cells did, however respond strongly to glutamine starvation. Although *E. coli* ASNase possesses a glutaminase side reaction, it is evident from the amino acid analyses that the steady state glutamine level remains at a marginal level in the medium, thus, complete withdrawal of glutamine may actually be a more severe starvation regimen than induced by the glutaminase side reaction of ASNase. Again, it appears that the potential for AS mRNA induction is greatly enhanced in resistant cells,

and that glutamine starvation, not ASNase treatment, is sufficient to elicit this response. Why the asparagine and glutamine starvation resulted in a synergistic enhancement of this response is unclear at this point.

For the resistant cells cultured in ASNase-free medium for 6 weeks, the AS mRNA response appeared to resemble aspects of both the parental and resistant cell lines. The response to asparagine starvation was more robust than the drug-maintained resistant cells, however glutamine starvation strongly enhanced AS mRNA levels (Figure 3-5, lanes 12-15). Taken together, these results are consistent with a cell line with a high capacity to up-regulate AS mRNA, but maintained in a medium environment that keeps the amino acid response generally repressed, resulting in extreme adjustments to AS levels when challenged with amino acid starvation.

The protein levels of AS during these experiments generally followed the mRNA expression with some exceptions (Figure 3-6). In particular, the glutamine starvations appeared to have a negative effect on AS protein synthesis as compared to the ASNase-induced AS mRNA expression in parental cells. This could be a result of low intracellular energy levels or reduced free glutamine available for protein synthesis.

To test whether the induction of AS is at a maximal level during ASNase treatment, parental and resistant MOLT-4 cells were incubated in the absence and presence of 1 U/mL ASNase in complete and histidine-free medium for 12 h. Cell extracts were isolated, and mRNA and protein levels were determined as for the previous experiments. Clearly, differences in the cell populations can be discerned, and these differences may have an affect on ASNase resistance. For the parental cells, there was no difference in the degree of AS mRNA induction between ASNase treated and histidine

Figure 3-5. Expression of Asparagine Synthetase mRNA During Glutamine and Asparagine Deprivation of MOLT-4 Cells. To assess the transcriptional regulation of AS during ASNase treatment, parental and resistant MOLT-4 cells (1×10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and subjected to northern analysis as described in Chapter 2. Also, cells were incubated in the absence of asparagine (-ASN), glutamine (-GLN), or a combination of both (-ASN, -GLN). The reversibility of AS mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis (Resistant 6 wk-ASNase). The blot (15 μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading (A). The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph (B). The data presented are representative of three independent experiments.

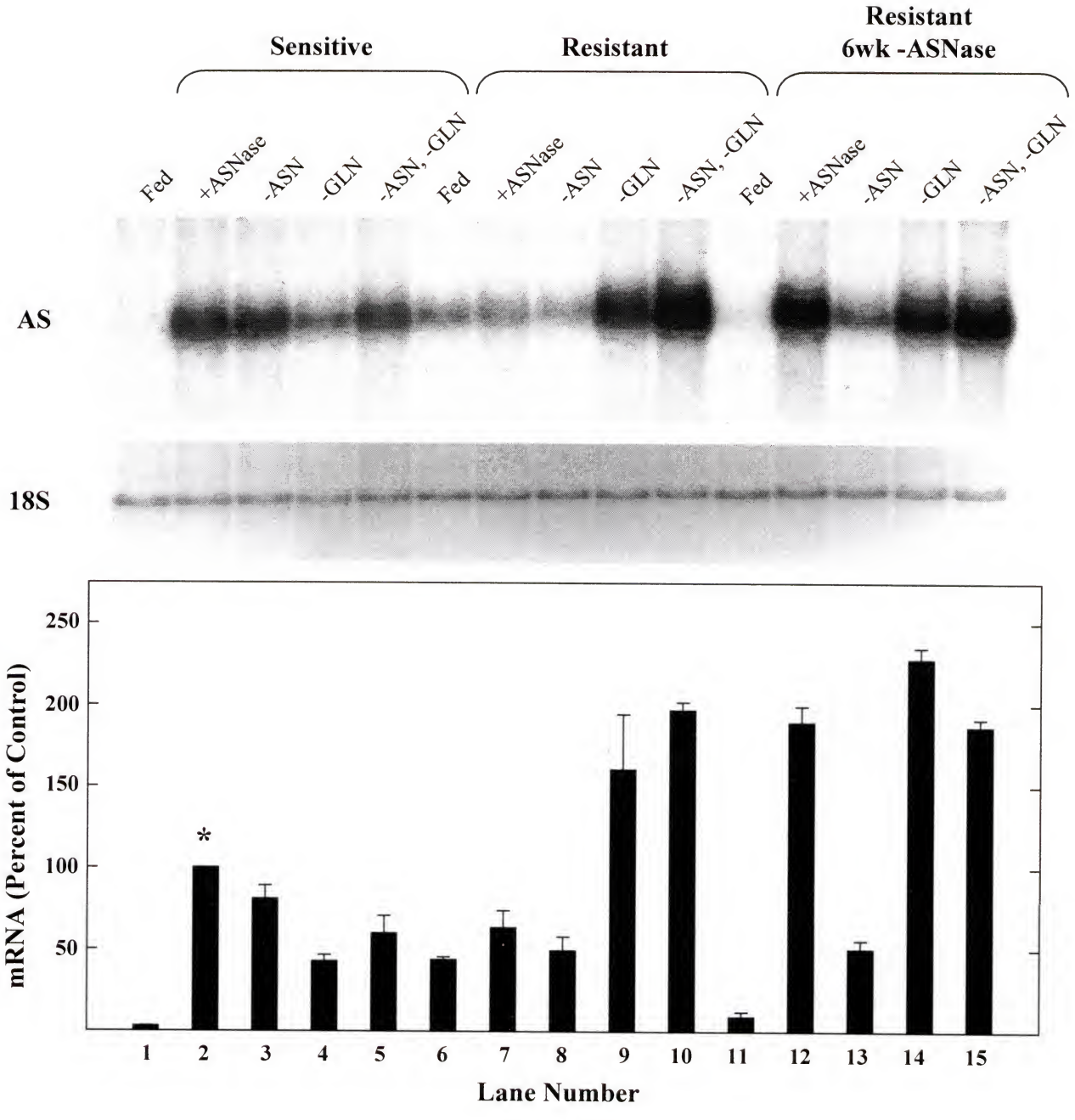

Sensitive
Resistant
Resistant
6wk -ASNase
Fed
+ASNase
-ASN
-GLN
-ASN, -GLN
Fed
+ASNase
-ASN
-GLN
-ASN, -GLN
Fed
+ASNase
-ASN
-GLN
-ASN, -GLN
AS
18S
mRNA (Percent of Control)
250
200
150
100
50
*
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Lane Number

Figure 3-6. Expression of Asparagine Synthetase Protein During Glutamine and Asparagine Deprivation of MOLT-4 Cells. To determine the regulation of AS protein in response to ASNase treatment, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and subjected to immunoblot analysis as described in Chapter 2. Also, cells were incubated in the absence of asparagine (-ASN), glutamine (-GLN), or a combination of both (-ASN, -GLN). The reversibility of AS mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis (Resistant 6 wk-ASNase). The blot (40 μg/lane) was probed with a monoclonal antibody to AS and visualized with a goat anti-mouse HRP secondary antibody (A). The quantified data in this panel were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph (B). The data presented are representative of three independent experiments.

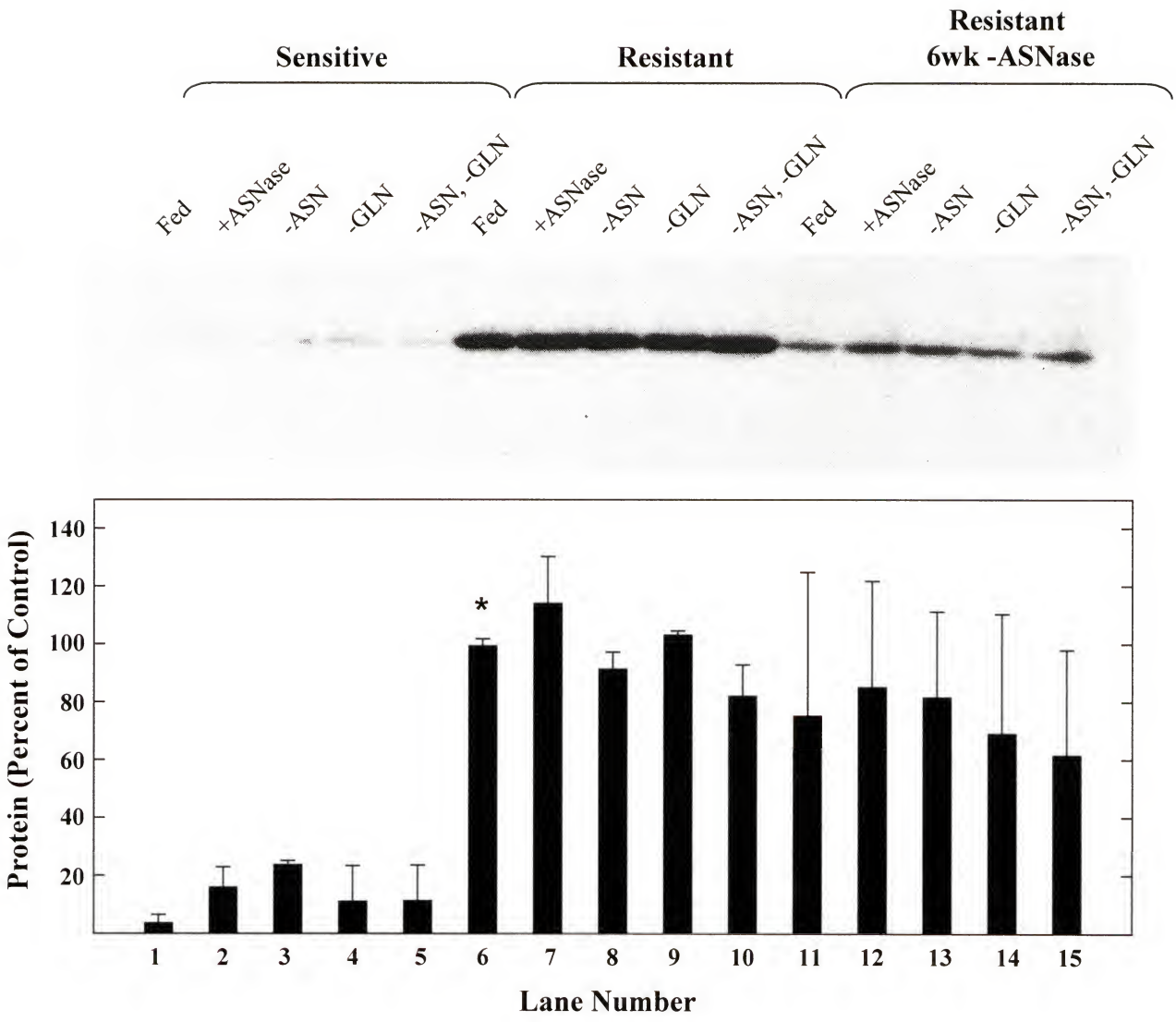

Sensitive
Resistant
Resistant
6wk -ASNase
Fed
+ASNase
-ASN
-GLN
-ASN, -GLN
Fed
+ASNase
-ASN
-GLN
-ASN, -GLN
Fed
+ASNase
-ASN
-GLN
-ASN, -GLN
Protein (Percent of Control)
140
120
100
80
60
40
20
*
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Lane Number

Figure 3-7. Expression of Asparagine Synthetase mRNA During Histidine Deprivation of MOLT-4 Cells. To assess the transcriptional regulation of AS during ASNase treatment, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and subjected to northern analysis as described in Chapter 2. Also, cells were incubated in the absence of asparagine (-HIS), or a combination of histidine depletion and ASNase (-HIS, +ASNase). The reversibility of AS mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis (Resistant 6 wk-ASNase). The blot (15 μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading (A). The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph (B). The data presented are representative of three independent experiments.

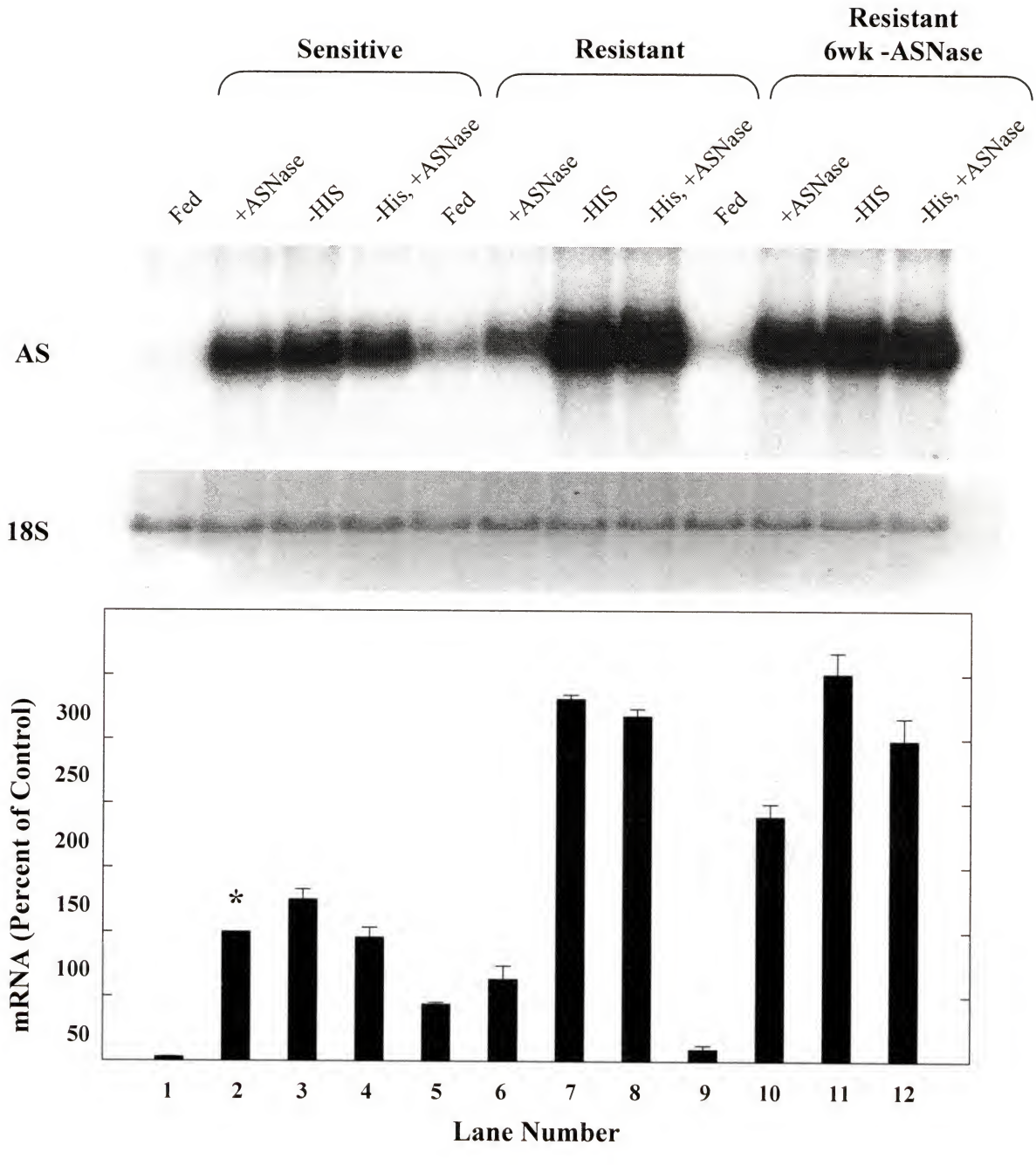
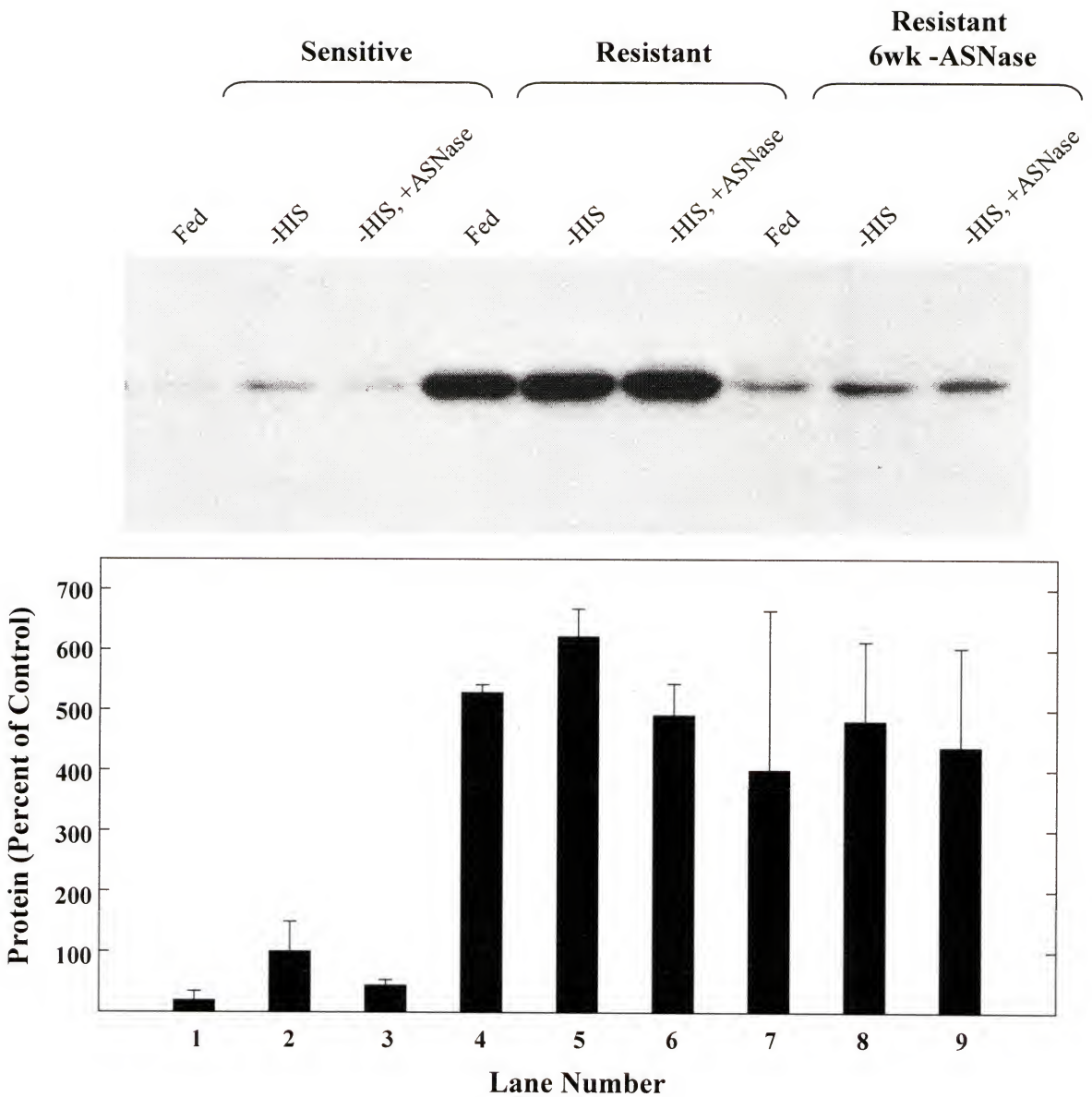

Sensitive
Resistant
Resistant
6wk -ASNase
Fed
+ASNase
-HIS
-His, +ASNase
Fed
+ASNase
-HIS
-His, +ASNase
Fed
+ASNase
-HIS
-His, +ASNase
AS
18S
mRNA (Percent of Control)
300
250
200
150
100
50
*
1
2
3
4
5
6
7
8
9
10
11
12
Lane Number

Figure 3-8. Expression of Asparagine Synthetase Protein During Histidine Deprivation of MOLT-4 Cells. To determine the regulation of AS protein in response to ASNase treatment, parental and resistant MOLT-4 cells (1×10^4 cells/mL) were incubated in the absence (control) or in the presence (+ASNase) of 1 U/mL ASNase for 12 h and subjected to immunoblot analysis as described in Chapter 2. Also, cells were incubated in the absence of asparagine (-HIS), or a combination of histidine depletion and ASNase (-HIS, +ASNase). The reversibility of AS mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis (Resistant 6 wk-ASNase). The blot (40 μg/lane) was probed with a monoclonal antibody to AS and visualized with a goat anti-mouse HRP secondary antibody (A). The quantified data in this panel were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph (B). The data presented are representative of three independent experiments.


Sensitive
Resistant
Resistant
6wk -ASNase
Fed
-HIS
-HIS, +ASNase
Fed
-HIS
-HIS, +ASNase
Fed
-HIS
-HIS, +ASNase
Protein (Percent of Control)
700
600
500
400
300
200
100
1
2
3
4
5
6
7
8
9
Lane Number

depleted cells (Figure 3-7). Given that histidine deprivation has been documented to elicit the maximum AS expression in FAO cells (Hutson et al., 1997), it appears that the AS response to ASNase in parental cells is at a maximal level, implying that perhaps the asparagine needs of the cell outstrip the capacity of the cell to respond to ASNase treatment. The resistant cells, on the other hand, not only have a higher capacity for up-regulation of AS as shown in the histidine depleted lane, but have a response to ASNase that is a fraction of this maximal response. The fact that resistant cells do not express AS at maximal levels during routine culture in ASNase-containing medium indicates that their need to up-regulate AS is within the dynamic range of the cell, perhaps giving them a selective advantage in amino acid depleted medium. As with the previous glutamine experiment, the protein levels of AS generally follow the AS mRNA expression, however, ASNase treated cells cultured in the absence of histidine have lower levels of AS protein than the cells starved for histidine alone, perhaps as a result of reduced protein synthesis due to the absence of both glutamine and asparagine.

Overexpression of AS and ASNase Resistance

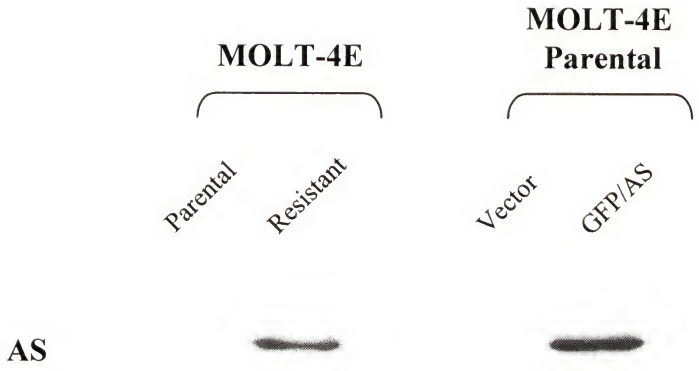
To determine if the enhanced expression of AS is solely responsible for the ASNase-resistant phenotype, the AS gene was expressed in parental MOLT-4 cells and an ASNase challenge assay performed on both AS-transduced and vector only control cells. To express AS, a Moloney mouse leukemia virus (MoLV) system was utilized, which required the transfection and selection of MOLT-4 cells with the murine viral receptor, CAT-1. These cells are designated MOLT-4 "ecotropic" receptor expressing cells (MOLT-4E), and were used in subsequent infections with the AS-containing virus. To determine the amount of AS expression in the AS transduced MOLT-4E cells versus

the vector only control, as well as to compare the AS expression between ASNase resistant MOLT-4E cells and parental MOLT-4E cells, a western blot probed for AS is shown in figure 3-9A. To monitor the efficiency of viral infection, and to sort for AS overexpressing cells, a MoLV viral vector was utilized that had an IRES and GFP coding region downstream of AS. Figure 3-9B shows the expression of GFP in AS/GFP co-expressing cells after infection and two rounds of FACS sorting as compared to non-infected MOLT-4E cells. As indicated, 76% of the AS/GFP cells are GFP positive when comparing to a non-infected control. To establish whether G418 selection resulted in cross-resistance to ASNase, an apoptosis assay was performed on non-infected parental MOLT-4 cells (Figure 3-9C). The data indicate that no cross-resistance was acquired by long-term selection in G418.

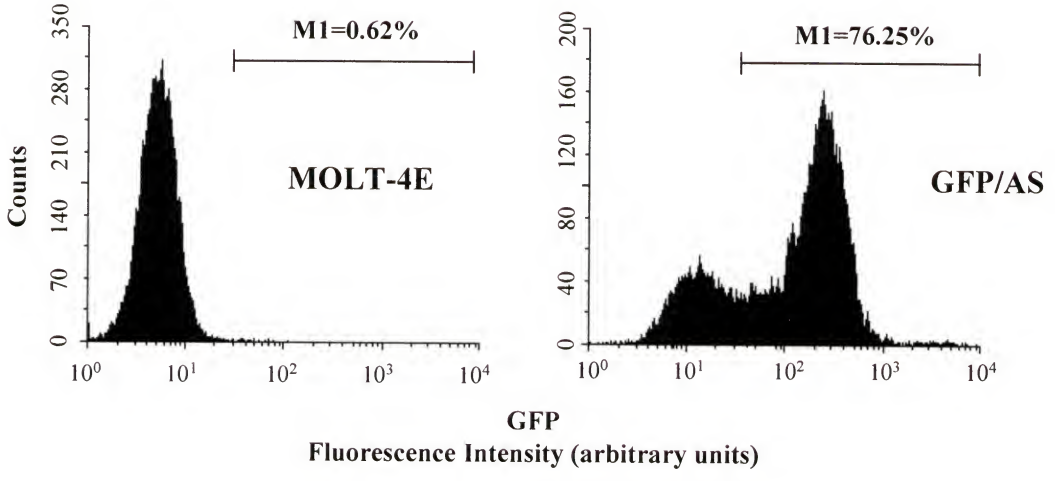
To determine if the expression of AS in parental MOLT-4 lymphocytes protects the cells from ASNase-induced cytotoxicity, an ASNase challenge assay was performed by incubating AS/GFP and GFP/vector only transduced cells in the absence and presence of 1 U/mL ASNase for 48 h, upon which a WST-1 assay (Figure 3-10A) and an annexin-V apoptosis assay were carried out (Figure 3-10B). The data in Figure 3-10A indicate that, in the presence of ASNase, the growth of MOLT-4E cells expressing AS is comparable to resistant MOLT-4E cells. Also, as shown by the FACS apoptosis analysis in figure 3-10B, the overexpression of AS in parental MOLT-4E cells results in a reduction of ASNase-induced cytotoxicity as compared to GFP/vector only control cells (74.2% vs. 25.7%). Taken together, these data indicate that expression of AS in otherwise sensitive parental MOLT-4 cells results in a significantly enhanced resistance to ASNase treatment. Although some apoptosis and cell death was observed in AS

Figure 3-9. Overexpression of AS in MOLT-4E Lymphocytes. To determine the extent of AS expression in AS/GFP transduced cells and to compare levels of AS expression between non-transduced MOLT-4 resistant cells and AS/GFP transduced cells, MOLT-4E parental, resistant, vector transduced and AS/GFP transduced cells were subjected to western blot analysis as described in Chapter 2 (A). To determine the percentage of cells expressing GFP in the AS/GFP transduced cells, FACS analysis comparing non-infected MOLT-4E cells and AS/GFP transduced cells was performed as described in Chapter 2 (B). The percentage of GFP-positive cells was determined from a total of 10,000 counted. To determine the sensitivity of G418-selected MOLT-4E cells to ASNase, an annexin V-PE apoptosis assay was performed as described in Chapter 2 (C). The numbers in each quadrant indicate the percentage of cells from a total of 10,000 counted. The data presented are representative of two independent experiments.

A


MOLT-4E
MOLT-4E Parental
Parental
Resistant
Vector
GFP/AS
AS

B


M1=0.62%
MOLT-4E
M1=76.25%
GFP/AS
Counts
GFP
Fluorescence Intensity (arbitrary units)

C

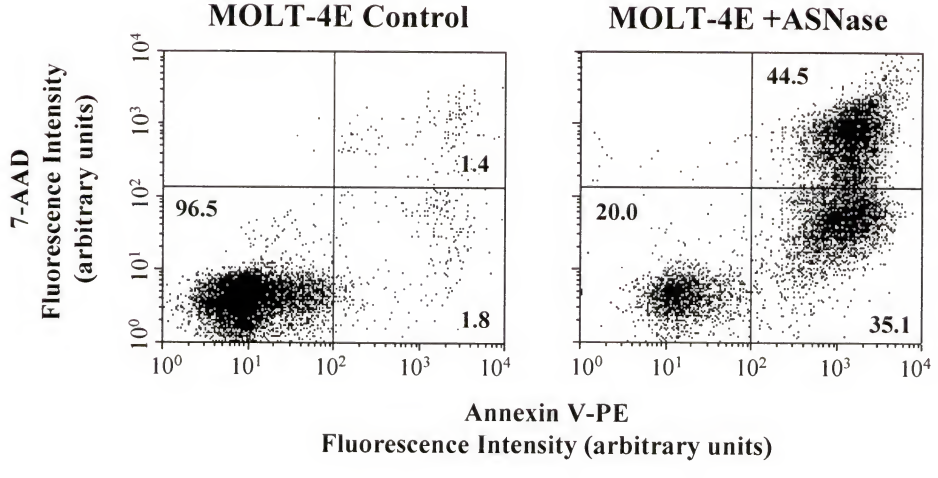
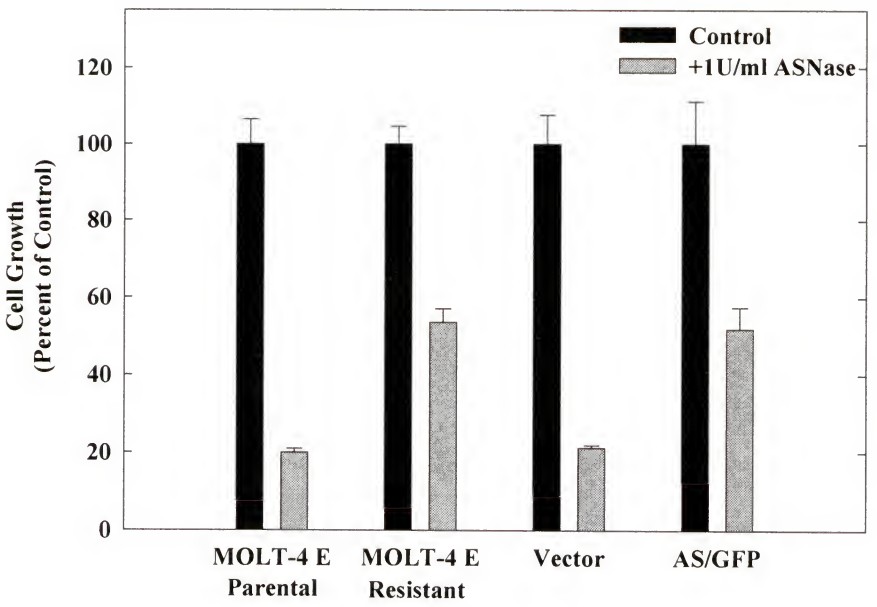
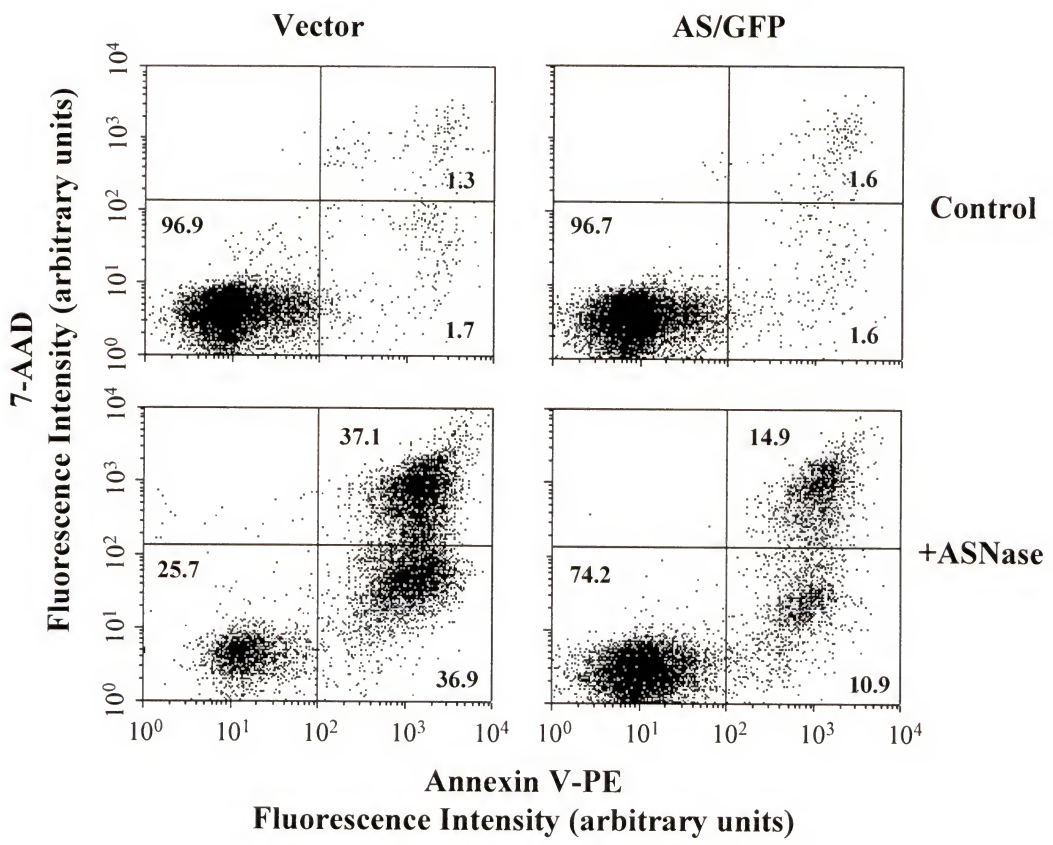

MOLT-4E Control
MOLT-4E +ASNase
7-AAD Fluorescence Intensity (arbitrary units)
96.5
1.4
1.8
44.5
20.0
35.1
Annexin V-PE
Fluorescence Intensity (arbitrary units)

Figure 3-10. Effect of ASNase on Cell Growth and Viability in AS-Transduced MOLT-4E Cells. To determine the effect of ASNase on cell growth and viability in AS overexpressing cells, AS and vector only transduced MOLT-4E cells (1 x 10^4 cells/mL) were incubated for 48 h in the absence (control) or presence (+ASNase) of 1 U/mL ASNase and performing a WST-1 cell proliferation assay (A) and an annexin V-PE apoptosis assay (B) and as described in Chapter 2. For the WST-1 cell growth assay, the absorbance of the treated cells is plotted as a percentage (± SD, n=4) of the untreated control for each individual cell line. For the apoptosis analysis, the numbers in each quadrant indicate the percentage of cells from a total of 10,000 counted. The data presented are representative of two independent experiments.

A


Control
+1U/ml ASNase
Cell Growth
(Percent of Control)
120
100
80
60
40
20
0
MOLT-4 E
Parental
MOLT-4 E
Resistant
Vector
AS/GFP

B


Vector
AS/GFP
Control
+ASNase
96.9
1.3
1.7
96.7
1.6
1.6
25.7
37.1
36.9
74.2
14.9
10.9
7-AAD
Fluorescence Intensity (arbitrary units)
Annexin V-PE
Fluorescence Intensity (arbitrary units)

transduced cells incubated in the presence of ASNase, the sorting analysis in figure 3-9B indicates that approximately 75% of GFP/AS transduced cells do not express GFP. It is likely that the expression of AS must be above a threshold level in order to afford the cells protection from ASNase cytotoxicity. Although a sub-population of cells may not express AS to high levels, the significant protection of AS transduced cells as compared to GFP/vector only control cells from ASNase-induced apoptosis and cell death indicates that, in some cells, overexpression of AS alone is sufficient for ASNase resistance.

Discussion

ASNase treatment of parental MOLT-4 lymphoblasts has profound effects on the growth and apoptosis of these cells, consistent with previous studies that showed that MOLT-4 cells are among the most susceptible cell type to ASNase treatment (Koishi et al., 1984; Hutson et al., 1997). Parental cells treated with 1 U/mL of the drug for 48 h exhibited growth arrest and cell death, with 64% of the cells either necrotic or undergoing apoptosis.

The precise mechanism of ASNase cytotoxicity is not known, although the inhibition of protein synthesis due to a lack of intracellular asparagine is thought to be a major factor in the effectiveness of the drug as an anti-neoplastic agent. This hypothesis has been generated from the observation that protein synthesis in Gardner lymphosarcoma 6C3HED cells is blocked when deprived of extracellular asparagine (Sobin and Kidd, 1965; Schwartz, 1963). Ellem et al. (1970) also have documented that 6C3HED cells undergo a rapid decrease in protein synthesis, followed temporally by a depression in the rate of DNA synthesis, rRNA synthesis, and tRNA synthesis upon

ASNase treatment. The effect of nutritional deprivation of asparagine of these cells resulted in a similar sequence of inhibition, although somewhat less rapid than ASNase treatment, with a half-life protein synthesis decline of 9 h as compared to a biphasic response with an initial half-life of 11 min, followed by a steady decline with a half-life of 84 min when treated with guinea pig ASNase.

The blockade of DNA synthesis as a result of protein synthesis repression also results in an inhibition of the cell cycle, which may, in turn lead to apoptosis. Cell cycle studies on bone marrow aspirates from ALL patients indicated that ASNase treatment for 48 h preferentially killed proliferative blasts and prevented progression of cells into the S phase of the cell cycle (Saunders, 1972). Cells that were previously engaged in S phase, however, continued to mitosis, such that no decrease in the number of cells in M phase was detected until 24 h after treatment (Saunders, 1972). Also, murine L5178Y leukemia cells were arrested in G1 phase upon ASNase treatment, with fragmentation of chromosomal DNA observed in the G1 arrested cells as early as 8 h after drug treatment (Ueno et al., 1997). The observations of cell cycle blockade as a result of ASNase treatment are consistent with the role of AS as a cell cycle related gene. As discussed in the introduction, Basilico et al. (1987), originally isolated the human AS gene as a clone that relieved a G1 block in BHK cells, indicating that low intracellular asparagine levels can serve as a signal for G1 arrest. The results obtained for the MOLT-4 lymphocytes seem to be consistent with these studies, indicating that ASNase induces both cytotoxic and cytostatic effects on these cells.

An alternative hypothesis for cell toxicity of ASNase suggested by Ryan and Dworak (1970) was based on the observation that in the 6C3HED lymphosarcoma,

glycine was preferentially depleted in sensitive cells exposed to ASNase as compared to resistant cells, which had increased glycine content after drug treatment. The authors suggested that transamination of the reactive glyoxylate species with asparagine, yielding α-ketosuccinamate and glycine, was an important pathway in cell detoxification that was perturbed in cells exposed to ASNase. However, studies by Keefer et al. (1985) in the LY5178Y lymphoma revealed an unexpected reduction in the intracellular glycine content of both sensitive and resistant cells upon ASNase treatment, leading to the conclusion that this effect was cell-type specific. For the MOLT-4 cells, the data in Table 3-1 indicate a doubling of the glycine content of both parental and resistant cells after ASNase treatment. Whether this increase is due to increased glycine synthesis via transamination of glyoxylate and asparagine, or enhanced transport due to the up-regulation of the amino acid transport Systems A and ASC (Chapter 4) is unclear.

In contrast to the effects on parental MOLT-4 cells, ASNase did not induce apoptosis in drug-selected resistant cells, although there was a growth slowdown of approximately 30% in the cells maintained in ASNase relative to drug-selected resistant cells incubated in the absence of ASNase for 48h. The ability of resistant cells to escape ASNase selection has been the subject of investigation ever since ASNase has been used in the treatment of leukemia. Broome (1968) first described the metabolic differences between sensitive and resistant 6C3HED tumors, documenting that resistant cells had a greater AS activity than sensitive tumors. This observation was extended by Prager and Bachynsky (1968), who identified four ASNase resistant mouse lymphomas with significantly higher AS activity than sensitive tumors. Clinical studies in human patients, however limited, have verified the circumstantial relationship between increased AS

activity and ASNase resistance. Haskell and Canellos (1969) identified five patients resistant to ASNase therapy that exhibited increased AS activity as compared to 4 patients that were successfully treated with the drug. In terms of *in vitro* cell cultures, a human lymphoma U937 cell line made resistant to ASNase by selection in increasing concentrations of the drug resulted in an 80-fold increase in AS activity (Kiryama et al., 1988). Similarly, ASNase resistant MOLT-4 cells have at least a 7-fold increase in AS activity as compared to parental cells (Hutson et al., 1997).

The data presented in Figures 3-2 and 3-3 indicate that resistant MOLT-4 cells maintained in 1 U/mL ASNase have a clearly increased AS mRNA content relative to parental cells (lanes 1 and 4). Also, the protein levels of AS are enhanced approximately 14-fold in resistant cells as compared to parental cells (Figure 3-4). Interestingly, upon ASNase treatment for 12 h, parental cells have an increase in AS mRNA that exceeds that of the resistant cells, however, the AS protein level is maintained at a relatively low level (Figure 3-4, lane 2). These results indicate that, although the adaptive mechanism for AS mRNA induction is present in the parental cells, the ability to translate this mRNA into AS protein is less efficient, perhaps, paradoxically, as a result of decreased asparagine available for protein synthesis. These data are similar to the observations of Prager and Bachynsky (1968), who observed a markedly elevated AS activity that was maintained at a high level in resistant mouse lymphomas, whereas sensitive cells exhibited a smaller, transient increase in AS activity that gradually declined until cell death.

The amino acid starvation-dependent expression of AS mRNA is actinomycin D and cycloheximide sensitive, indicating that the synthesis of a trans-acting factor may be necessary for AS expression (Hutson and Kilberg, 1994). Given that the transcription of

a number of genes is affected by amino acid starvation, including ODC, ribosomal proteins L17 and S25, and AS (Marten et al., 1994), the putative trans-acting factor may act in a "general control" response, similar to the yeast transcription factor GCN4. Although the transcriptional response of AS to asparagine starvation is active in MOLT-4 lymphocytes within 12 h, it is possible that sensitive cells are unable to maintain the production of AS mRNA at later time points because of impaired transcription factor synthesis.

Since the short-term nutrient adaptive mechanisms for AS appear to be functional for parental MOLT-4 cells, perhaps the most significant factor in the ability of the resistant cells to adapt to the challenge of ASNase treatment may be the increased basal level of AS expression. The elevated AS mRNA and protein content at the onset of ASNase exposure may give the cell an enhanced capacity to synthesize additional AS, providing a selective advantage during asparagine deprivation. Clearly, this enhanced expression is a long-term genetic modification of resistant cells, because resistant MOLT-4 lymphocytes cultured in the absence of ASNase for 6 weeks have elevated levels of AS mRNA and protein (Figures 3-2,3-3,3-4). Furthermore, these cells appear to retain phenotypic characteristics of resistant cells maintained in ASNase, exhibiting little to no apoptosis upon ASNase challenge (Figure 3-1). The ability of ASNase resistant lymphocytes to permanently up-regulate AS has also been demonstrated by Kiryama et al. (1989), who documented that the AS activity of ASNase resistant U937 cells remained at a level 80-fold that of sensitive cells after 6 months of growth in medium containing a 5-fold excess of extracellular asparagine. Unfortunately, this group did not perform

ASNase challenge assays on these cells to monitor ASNase resistance after extended asparagine repletion.

The fact that basal AS expression may be the most important factor in ASNase resistance is demonstrated by the data presented in figures 3-8 and 3-9, which indicate that overexpression of AS alone in sensitive cells results in a significant reduction of ASNase-induced cytotoxicity. Unfortunately, since the sorted cell population used in the challenge assay includes cells with a wide range of GFP/AS expression, it is not clear whether the cells undergoing apoptisis and necrosis in the AS overexpressing cells are affected because of insufficient AS expression, or other metabolic factors. In spite of this caveat, it is clear that in a subpopulation of sensitive cells, complete ASNase resistance may be conferred by the overexpression of AS. These results also coincide with the experiment in figure 3-7, that indicates ASNase exposure elicits a maximal AS response in parental MOLT-4 cells which is likely not adequate to maintain protein synthesis. Conversely, resistant MOLT-4 cells have a sufficient basal expression of AS to maintain cell viability as well as an additional capacity to transcriptionally up-regulate the gene as evidenced by the histidine deprivation. Taken together, these data indicates that ASNase treatment initiates cell death before the cell can adaptively up-regulate AS to sufficient levels to continue protein synthesis and maintain cell viability, and that overexpression of AS alone prevents this ASNase-induced cytoxicity.

Although AS overexpression may protect cells from acute ASNase treatment, it is essential that resistant cells maintain this high level of expression to remain resistant. In terms of the mechanism of long-term AS up-regulation, gene amplification of AS has been shown in several studies not to be associated with ASNase resistance (Martin et al.,

1993; Andrulis et al., 1990), however, there is considerable circumstantial evidence that an alteration in the methylation of the AS gene may contribute to the long-term enhanced expression of AS in resistant cells. Methylation is now becoming recognized as an important factor in tumor progression, with the discovery of hyper- and hypo-methylated genes resulting in the silencing and reactivation, respectively, of genes that influence cellular proliferation and drug escape. A clinical study comparing the methylation status of bone marrow aspirates from normal and acute myelogenous leukemia (AML) patients revealed that the calcitonin, estrogen receptor, E-cadherin, tumor suppressors p15 and p16, Rb, GST-Pi or HIC1 genes were abnormally methylated in 95% of AML patients as compared to complete demethylation of all genes for the normal patients (Melki et al., 1999). Conversely, selection of cells in vincristine or doxorubicin often results in the overexpression of multidrug resistance gene 1 (MDR1), which encodes the cell surface P-glycoprotein. Nakayama et al. (1998) have identified an inverse correlation between the methylation status of the MDR1 promoter and MDR1 expression, indicating that hypomethylation of this gene may be important for MDR1 overexpression and the subsequent generation of p-glycoprotein mediated multidrug resistance.

The AS gene has also been shown to be modulated by methylation in several *in vitro* studies. Asparagine auxotrophic JRS cells could be rendered asparagine independent by treatment of the cells with the nucleoside analog, 5-azacytidine, which effectively results in global demethylation, and accordingly, significantly enhanced AS expression (Andrulis et al., 1987). Also, selection of CHO cells with ASNase resulted in an extensive demethylation of the 5' and 3' regions of AS, corresponding with an enhanced expression of the gene. Perhaps one of the most interesting observations

regarding the relationship between AS expression and methylation is the demonstration by Andrulis et al. (1991) that ASNase treatment alone caused a global reduction in methylation of 25% in CHO cells. This indicates that ASNase treatment may affect long-term gene expression by acting as a demethylating agent as well as a selective agent for the appropriate phenotype, however, with the observation that tumor progression in general is associated with methylation abnormalities, it is likely that ASNase is not necessarily the only factor in inducing the hypomethylation of AS.

Although increased AS expression has been presumed to be the mechanism which renders cells resistant to ASNase, and in fact, AS overexpression in sensitve cells significantly reduces ASNase-induced cytotoxicity, other metabolic perturbations may be important in the maintenance of complete ASNase resistance. Broome (1968) observed that resistant cells efficiently utilized intracellularly produced asparagine, allowing them to continue protein synthesis even at free asparagine levels below that of ASNase treated sensitive cells. This observation led to the speculation that there existed a source of asparagine that was preferentially "channeled" towards protein synthesis. The intracellular amino acid data of Table 3-2 indicate that resistant MOLT-4 cells have a concentration of free asparagine below detectable levels, yet they continue to proliferate, albeit at a slightly reduced rate than untreated controls. These data indicate that the resistant MOLT-4 cells may have a mechanism of providing asparagine synthesized directly towards protein synthesis, similar to the model suggested by Broome (1968) or that the flux of asparagine from AS to utilization is greater in resistant cells, but is consumed at a level such that no accumulation of free asparagine occurs.

Taken together, the data presented in this chapter give several novel insights as to the mechanism of ASNase treatment and the expression of AS in response to drug therapy. First, ASNase resistant cells have all the transcriptional machinery to up-regulate AS, refuting the hypothesis that cells are ASNase sensitive due to the lack of gene induction. Parental MOLT-4 cells also may be rendered resistant to ASNase by overexpression of AS, indicating that apoptosis and necrosis initiates before the cell may adaptively up-regulate AS. Furthermore, although acute ASNase-induced cytotoxicity may be prevented by AS overexpression, MOLT-4 resistant cells have a long-term component of AS up-regulation that maintains the resistant phenotype in the absence of ASNase.

CHAPTER 4
TRANSPORT AND METABOLISM OF ASPARAGINE SYNTHETASE SUBSTRATES

Introduction

As discussed in Chapter 3, MOLT-4 lymphocytes cultured in increasing concentrations of ASNase become resistant to the drug and exhibit significant up-regulation of AS as compared to parental cells. Considering that the level of AS protein is highly induced in these cells, it follows that glutamate and aspartate must be provided in sufficient quantity to supply the enzyme with ample substrate. The cell may either increase the influx of glutamine and aspartate from the extracellular milieu, or increase the synthesis of these amino acids *de novo* via glutamine synthetase (GS) or transamination reactions to maintain asparagine production and, accordingly, ASNase resistance.

The primary mode of glutamine entry into lymphocytes is mediated transport via a series of plasma membrane transporters with overlapping substrate specificity and regulatory properties. The amino acid transport systems identified in lymphocytes share the classically defined characteristics of transporters described for many tissues, however, a distinction should be noted between peripheral human lymphocytes, which are primary venous blood samples that undergo a Ficoll lymphocyte separation, and may consist of a heterogeneous mixture of B-and T-lymphocytes having distinct transport characteristics, and lymphoblast cell lines which consist of a single B- or T-lymphoblast cell type. Given this potential complication, the major zwitterionic amino acid transport agencies

identified in lymphocytes are quite limited, being restricted to System A, System ASC and System L.

Originally described in the Ehrlich Ascites cell by Christensen and Oxender (1963), System A is a ubiquitous sodium-dependent transport system that has been identified in every nucleated cell tested to date. System A has been identified in human peripheral lymphocytes, human lymphoblast cell lines, porcine lymphocytes, mouse lymphocyte cell lines, rat splenocytes and rat thymocytes (Segel et al., 1983; Bass and Englesberg, 1979; Borghetti et al., 1981; Dantzig, et al., 1979; Wise, 1978). The substrate specificity of this carrier includes small, unbranched zwitterionic amino acids such as alanine, cysteine, and glutamine, however, many amino acids have a percentage of their total uptake mediated by System A. A distinguishing feature of System A is the toleration of a monomethylated α-amino group, thus the activity of this transport system may be measured by inhibition of test substrates by the non-metabolizable alanine analog, 2-(methylamino)-isobutyric acid (MeAIB) (Kilberg et al., 1983). Another unique characteristic of System A is an exquisite pH dependence, which results in a significant loss of transport capacity below a pH of 7.0 (LeCam and Freychet, 1977; Kilberg et al., 1980). The titration of an ionizable histidine group is postulated to be responsible for this loss of activity, however, this hypothesis has only been extrapolated from the observed range of pH sensitivity, and has not been experimentally verified. In terms of the alkali-ion dependence of System A, the Ehrlich cell demonstrates the ability to accept Li^+ instead of Na^+ as the cationic species necessary for symport of the amino acid, however, freshly isolated hepatocytes appear to be intolerant of Li^+ substitution (Christensen and

Handlogden, 1977; Edmondson, et al., 1979). Currently, the alkali-ion dependence of System A in lymphocytes is unknown.

The kinetic parameters of System A appear to be largely cell type dependent. Many monolayer cell cultures exhibit a high affinity (~1 mM) Km for System A, whereas freshly isolated hepatocytes also include a low affinity (~20 mM) Michaelis constant (Km) component that is absent after 20 h of culture (LeCam and Freychet, 1977; Kelly et al., 1982; Morin et al., 1979). Lymphocytes exclusively express the high affinity System A component, with reported Km values for human peripheral lymphocytes between 500 μM and 2 mM (Segel et al, 1983; Bass and Englesberg, 1979). Similarly, mouse L5178Y lymphocytes have a measured Km of 0.89 mM for System A (Finklestein and Adelberg, 1977).

Two distinct System A genes (ATA1 and ATA2) have been recently cloned, each with a specific tissue distribution. The original ATA1 clone was discovered by screening a rat neuronal cDNA library with a region of a mouse expressed sequence tag (EST) that exhibited homology to the mammalian vesicular glutamate and γ-isobutyric acid (GABA)/glycine transporter as well as the conserved RUNC-1 region of plant, *C. elegans*, and yeast amino acid permeases. The cDNA that was obtained revealed MeAIB-inhibitable transport consistent with the classically described substrate specificity of System A when transfected into CV cells (Varoqui et al., 2000). Whereas ATA1 is neuronal specific, the expression of ATA2 appears to be widespread, with 4.7 and 2.5 Kb mRNA transcripts identified in brain, thymus, lung, heart, muscle, intestine, liver, kidney, testis, spleen, and skin. The ATA2 cDNA was cloned by low-stringency screening of a rat skeletal muscle library with a probe generated from the neuronal-specific glutamine

transporter ATA1, and shares a 55% identity with ATA1, encoding a 504 amino acid, 11 transmembrane domain protein that exhibits the substrate specificity, sodium ion dependence, and pH sensitivity consistent with System A (Sugawara et al., 2000).

An important characteristic of System A is the ability of this carrier to undergo significant adaptive and hormonal regulation. The dependence of System A on the cell cycle was originally described in the regenerating liver, however, polyclonal mitogens such as plant lecithins are useful in determining the cellular changes that stimulate DNA, RNA and protein synthesis during proliferation in lymphocytes. The activation of human peripheral lymphocytes with the mitogenic agent phytohemagglutinin (PHA) results in a 4-fold increase in aminoisobutyric acid (AIB), alanine, and proline uptake after a 4 h exposure (Segal and Lichtman, 1981), however, the non-mitogenic agent wheat germ agglutinin had a negative effect on System A transport (Greene at al., 1976). This response does not affect the Km of System A, rather, it appears that there is a requirement for *de novo* protein synthesis of new transport proteins, based on studies which employed cycloheximide and actinomycin D during PHA exposure (Segal and Lichtman, 1981, Borghetti et al., 1981). Interestingly, this adaptation could be superimposed on an increase of System A in response to amino acid deprivation, implying that separate signaling pathways are responsible for enhanced activity. Additionally, the half-life of System A decay is extended from 4 to 8 h after lecithin stimulation in the presence of cycloheximide, indicating that transporter turnover also is affected by mitogenic agents (Borghetti and Kay, 1979).

Clearly, System A adaptation by amino acid limitation is of particular importance in terms of ASNase therapy. The phenomenon of transport adaptation to extracellular

amino acids was originally described in rat uterine and chick embryo cells incubated in amino acid-free medium (Gazzola et al., 1972). The enhanced transport observed was attributed to System A based on the pH sensitivity and substrate specificity of the heightened activity. This adaptation is present in lymphocytes, with increased System A activity observed in thymic lymphocytes and chronic lymphocytic leukemia (CLL) B-lymphocytes after incubation in amino acid-free medium (Peck et al., 1976; Frenghley et al., 1975). These transport increases were mRNA and protein synthesis dependent, consistent with System A adaptation in many other cell types. Interestingly, blast cells from ALL patients exhibited a greater increase of transport activity as compared to cells from CLL patients. Also, cells undergoing large degrees of adaptation did not completely suppress the adaptive response (Frenghley et al., 1975).

Another major transport system identified in human peripheral lymphocytes and chronic leukemia lymphoblasts is System L. This carrier functions independently of the sodium gradient, and is capable of heteroexchange, although a net flux of amino acid may be achieved by recycling of the transporter without a cytoplasmic amino acid. The substrate specificity of System L includes large, branched-chain and aromatic amino acids such as leucine, isolueucine, tyrosine, tryptophan, valine, phenylalanine, methionine and glutamine (Oxender and Christensen, 1963). In addition, substrates of System A become reactive at low extracellular pH levels (Guidotti et al., 1978). System L is intolerant of alkyl substitutions at the α-nitrogen, however, the activity of this system may be monitored indirectly by inhibition with the non-metabolizable model substrate 2-aminobicyclo(2,2,1)-heptane 2-carboxylic acid (BCH) (Christensen et al., 1969).

In terms of regulation, System L exhibits trans-stimulation, resulting in the acceleration of transport activity by intracellular substrates of System L. Furthermore, although early studies on the adaptive regulation of System L indicated that the activity of this system is unchanged in response to amino acid deprivation (Gazzola et al., 1972), an increase in the V*max* of System L transport was observed when a CHO cell line containing a temperature-sensitive mutant leucyl-tRNA synthetase was incubated at non-permissive temperature (Shotwell et al., 1982). This increase was actinomycin D insensitive, thus, in contrast to system A, which is regulated at the transcriptional level, System L appears to be regulated at the translational level by the ratio of charged to uncharged $tRNA^{Leu}$. Stimulation of human peripheral lymphocytes with the mitogenic lecithin PHA also enhances System L activity 2- to 3-fold, but only after a prolonged (24 h) exposure (Segel et al., 1981).

System L is significantly reduced in CLL B-cells, with a transport activity 15% that of blood lymphocytes, tonsillar lymphocytes, and normal B-lymphocytes (Segal and Lichtman, 1982). The divergence in transport rates between these cell lines could be reversed by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA), achieving levels of L-System transport comparable to normal B-cells after 40 h of exposure. This increase in System L was concomitant with the development of plasmacytoid features, indicating that the reduction of system L activity was a function of the immature CLL blast phenotype. Also, B-CLL cells are incapable of trans-stimulation of System L in the presence of high intracellular concentrations of BCH, however, TPA treatment also restored trans-stimulation of System L by intracellular substrates, consistent with the phenotype of mature B-cells (Segal et al., 1988).

Although System A and System L are expressed in different lymphocyte preparations to varying degrees, the bulk of zwitterionic amino acid transport in the majority of resting lymphocytes occurs via System ASC. A majority of the alanine uptake in human peripheral lymphocytes, and virtually all of the sodium dependent transport observed in porcine lymphocytes and rat splenic lymphocytes is mediated by System ASC (Segal et al., 1983, Borghetti et al., 1981, Wise, 1978). This activity, originally discovered when MeAIB failed to completely inhibit alanine uptake in the Ehrlich ascites cell, has a similar substrate specificity as System A, although it is highly stereoselective and intolerant of monomethylated α-amino acid derivatives such as MeAIB (Christensen et al., 1967). Also, in contrast to System A, System ASC is relatively insensitive to the extracellular hydrogen ion concentration, however, at pH levels below 6.0, anionic amino acids are capable of serving as substrates for this transport activity (Tamarappoo et al., 1996; Vadgama and Christensen, 1984). Based on the pH range of this substrate shift, it is postulated that titration of a histidine residue allows the binding of an anionic amino acid to the transporter, however, this has not been verified experimentally.

Reports of the ion dependence and kinetics of System ASC that appear in the literature are quite varied, and underscore the apparent mechanistic diversity that this transport system exhibits. Although linked to the Na^+-electrochemical gradient, System ASC appears to have an amino acid:Na^+ ion stochiometry that varies from one amino acid to four Na^+ ions in the case of proline, to four amino acids per Na^+ for homocysteine and proline. To reconcile these widely divergent coupling ratios, a model has been proposed by Koser, Christensen, and Thomas whereupon the relative preference of the

sodium ion to dissociate from the transporter when in the cytoplasmic orientation is dependent on the amino acid being co-transported (Thomas and Christensen, 1971; Koser and Christensen, 1971). Additionally, System ASC has been proposed to operate in a Na^+-dependent obligatory exchange mode, however, studies using a wide range of sodium and extracellular substrate concentrations have not, as of yet been published (Zerangue and Kavanaugh, 1996a).

The genes responsible for System ASC have been cloned, and it appears that this transport activity is encoded by two closely related variants (ASCT1 and ASCT2) with complementary tissue specificity (Arriza et al., 1993; Shafquat et al., 1993). ASCT1 was cloned by screening a human motor cortex library using a degenerate oligonucleotide that corresponded to a conserved hydrophobic region from the evolutionarily related glutamate/aspartate transporters EAAT1-3. Subsequent expression of the ASCT1 cDNA in *Xenopus* oocytes and HeLa cells revealed a substrate specificity and ion dependence consistent with System ASC. The ASCT1 clone encodes a protein of 532 amino acids, with 6 transmembrane spanning domains, and is found in muscle, pancreas, brain, placenta, heart, lung, kidney, and liver. Northern analysis of tissues expressing ASC activity has revealed a series of three mRNA transcripts of 5.0, 3.5, and 2.2 Kb, representing variations of the 3' untranslated region (Arriza et al., 1993; Shafquat et al., 1993).

ASCT2 was isolated from a mouse testis library using degenerate RT-PCR primers designed against the ASCT1 sequence. This clone, when expressed in *Xenopus* oocytes, had a substrate specificity identical to that described for System ASC (Utsonomiya-Tate et al., 1996). Kekuda et al. (1996), performing low stringency

screening of a human placental choriocarcinoma cell line, obtained a clone that exhibited transport characteristics similar to that of the broad scope transport system B^o. The differences between these clones were resolved when screening of a mouse kidney library with the proposed ATB^o sequence revealed ASCT2 as the only positive clone, confirming that both genes were species variants of ASCT2. In terms of tissue distribution, ASCT2 is found in lung, intestine, kidney, skeletal muscle, testis, and white adipose tissue. Currently, there is no data on the mRNA expression of either ASCT1 or ASCT2 in lymphocytes.

The uptake of anionic amino acids in mammalian cells is primarily mediated by System $X_{A,G}^-$ and, to a much lesser extent, System x^-_c (Marvizon et al., 1981). System $X_{A,G}^-$ is defined as the D-ASP inhibitable, Na^+-dependent component of L-ASP or L-GLU uptake, owing to the strict stereoselectivity of the transporters that comprise this activity. System $X_{A,G}^-$-like activity is encoded by five distinct gene products (EAAT1-5), which appear to be evolutionarily related to the ASC subfamily, owing to a 40% similarity in amino acid sequence. Although all mediate the high affinity Na^+-dependent transport of L-ASP and L-GLU, each member of this transporter family exhibits a distinct tissue distribution and ontogenic expression pattern. EAAT3 appears to be the most widely distributed $X_{A,G}^-$ transporter in humans. The expression of this isoform has been identified in brain, intestine, kidney, heart, and liver tissue (Arriza et al., 1994; Kanai et al., 1995). EAAT1 appears to have a brain specific tissue distribution in rats, although in humans, peripheral tissues such as heart, lung, placenta and skeletal tissue also express this transporter (Kanai et al., 1995; Storck et al., 1992). EAAT2, EAAT4, and EAAT5

appear to be more restricted to brain (EAAT2, EAAT4) and retinal cells (EAAT5) (Pines et al., 1992; Fairman et al., 1995; Arriza et al., 1997).

Although the different isoforms share approximately 50-80% amino acid similarity, it appears that the alkali-ion dependence and transport characteristics between the members exhibit some heterogeneity. The mechanism of transport for EAAT3 involves the exchange of 3 Na^+ ions, 1 proton, and one anionic amino acid for one K^+ ion, whereas isoforms EAAT4 and 5 appear to function as amino acid activated Cl^- channels, indicating that chloride transfer, and not amino acid transport may be the primary physiological role of these isoforms (Zerangue et al., 1996a, Arriza et al., 1997).

In terms of regulation, members of the glutamate family have been reported to be differentially expressed in developing tissue. Blakely et al. (1991) used mRNA isolated from developing rat brain to demonstrate a region-specific distribution of EAAT transporters. In placental tissue, the ontogenic regulation of the transporters has been studied using antibodies and cDNAs to EAAT1-4. Placentas isolated from pregnant rats at day 20 of gestation have increased levels of EAAT1-4 as compared to day 14 placentas, however, each isoform was regulated differentially, with EAAT3 showing the greatest induction. Protein levels of the transporters also varied during development. Although a general trend towards increased expression was evident in day 20 placental tissues compared to day 14 for EAAT1 and 3, EAAT2 protein levels were reduced in the basal membrane and unchanged in the apical membrane (Matthews et al., 1998).

Differential regulation has also been observed for members of the EAAT family exposed to prostaglandins. In oocytes injected with either EAAT2 or EAAT1 cRNA, EAAT2 had an increase in substrate affinity, whereas EAAT1 transport was reduced by

approximately 30 percent after treatment of the cells with arachadonic acid (Smit et al., 1993). Also, amino acid dependent regulation of the EAAT family has been shown in response to amino acid starvation in bovine renal epithelial cells. Nicholson et al. (1996) has demonstrated that in response to complete amino acid starvation, Na^+-dependent L-ASP transport is increased approximately 1.5 to 3-fold, dependent on the confluency of the cell culture. The addition of single amino acids to the medium capable of conversion to L-GLU by transamination were able to reduce this induction by 50 percent. Interestingly, no increase in EAAT3 protein expression or mRNA levels was observed during the starvation, leading this group to propose the existence of an EAAT3 activating protein that up-regulates transport post-transcriptionally upon depletion of extracellular amino acids. However, these authors did not assay the cells for the presence and/or regulation of EAAT1, 2, 4, or 5 transporter proteins.

Although the substrate specificity for System x^-_c includes cysteine and glutamate, this exchanger generally functions in the export of glutamate, because intracellular cystine is rapidly reduced to cysteine, resulting in the net influx of cystine. This transport activity is mediated by two distinct proteins, one of which is identical to the heavy chain of the 4f2 cell surface antigen (4F2hc), and another novel protein named xCT. xCT is highly regulated, and its expression enhanced by stimulation of mouse peritoneal macrophages with lipopolysaccharide (Sato et al., 1999). Thus, xCT appears to be a member of a family of transporters which interact with 4F2hc in a heteromultimeric complex, each possessing different transport characteristics (Sato et al., 1999).

The literature regarding the expression of dicarboxylic acid transporters in lymphocytes is quite limited, however, from the few studies that have been conducted, it

appears that a considerable species and lymphocyte lineage heterogeneity may exist. In terms of anionic amino acid transport in human lymphocytes, System x^-_c has been identified in macrophages, however, little to no x^-_c transport activity was identified in resting or mitogenically stimulated MOLT-4 T-lymphocytes. Also, the addition of extracellular glutamate did not result in decreased intracellular cystine levels in MOLT-4 lymphocytes, whereas the pro-monooctyic U937 cells did exhibit a decrease in intracellular cystine after glutamate exposure, suggesting the inhibition of an active anionic transport system (Gmunder et al., 1991). In contrast to the results obtained for human T-lymphocytes, Dantzig et al. (1978) reported the uptake of glutamate in a mouse lymphocyte cell line derived from L5178Y by two distinct systems. Based on AIB inhibition and a transport mutant isolated by the tritium suicide method, it was concluded that 80% of this transport was mediated by System A, with the remainder of the glutamic acid transport in this cell line characterized as sodium-insensitive, with no inhibition by BCH or other System L substrates. These species and cell type differences in transport could have a dramatic impact on the interpretation of potential drug effects.

In addition to obtaining glutamine from the extracellular medium, lymphocytes also may catalyze the production of glutamine by GS. GS is found in prokaryotes and eukaryotes, and is termed GSI and GSII, respectively. Although there is a wealth of information on the structure, function, and regulatory properties of bacterial GSI, comparatively little is known about eukaryotic GSII. GSI is a dodecameric enzyme complex, formed by two hexameric rings of active subunits held together by hydrophobic and hydrogen bonding interactions (Almassy et al., 1986). The three-dimensional

structure of GSII is not known, although the core polypeptide consists of a 372-residue protein that shows distant similarity to GSI (Haschemeyer et al., 1968).

The regulation of GSI is primarily accomplished by negative feedback inhibition, with nine products of glutamine metabolism capable of inhibiting the GS biosynthetic reaction: serine, alanine, glycine, AMP, cytidine triphosphate (CTP), tryptophan, histidine, carbamoyl phosphate, and glucosamine-6-phosphate (Woolfolk et al., 1967). Although all the effectors of GSII have not been identified, the active sites are conserved between GSI and GSII, implying that the spectrum of inhibition may be similar, however, tissue-specific differences in GSII inhibition have been observed. Liver GSII is inhibited by glycine, alanine, serine, glutamine, histidine and carbamoyl phosphate in a Mn(II)-dependent manner. Liver GSII also is activated by α-ketoglutarate and citrate, however, brain GSII is inhibited by carbamoyl phosphate and does not respond to downstream metabolites derived from glutamine (Meister, 1974; Meister et al., 1984).

The differential regulation of GSII in brain and liver may be related to their functional roles in the appropriate tissues. Brain GS is primarily found in astrocytes, where glutamine is synthesized from neurotoxic ammonia and glutamate. The glutamine is then transported to neurons, where it functions as a precursor to the neurotransmitters GABA. Given the constant incorporation of glutamate into the cell from the synaptic junction, brain GS functions as a detoxifying and glutamate recycling agent in neurons. In liver and other tissues, GSII functions primarily as a biosynthetic enzyme for substrates of protein synthesis, and is therefore inhibited by feedback inhibition by glutamine-dependent metabolites. Given that the protein sequences for both the brain

and liver GSII are identical, significant post-translational modification is thought to account for the regulatory differences observed in these tissues.

The expression of GSII also is regulated by hormones. Glucocorticoids are the only systemic agents that have been shown to increase GS mRNA levels in muscle, gastrointestinal tract, lung, adipose, and lymphoid cells. However, hormonal treatment generally results in a significant GS mRNA induction with minimal protein response, further indicating that GS is primarily regulated by post-translational mechanisms in eukaryotic cells (Lie-Venema et al., 1998; Abcouwer et al., 1995; Ardawi., 1991; Lukaszewicz et al., 1997; Sarantos et al., 1993).

In terms of the mechanism of metabolite inhibition, down-regulation of GSII has been shown to occur in isolated cells by a process of protein destabilization. Increasing glutamine concentrations accelerate GSII protein decay in a variety of cell lines (Arad et al., 1976; Crook et al., 1978). Similarly, protein stabilization may function to increase GS levels during glutamine starvation. *In vivo* studies revealed that rats fed a low glutamine diet in conjunction with the GS inhibitor methionine sulfoximine (MSO) had a 50% reduction in plasma glutamine and a 70% reduction in lung glutamine levels as compared to glutamine-starved only rats. Under this rate-limiting synthesis condition, lung tissue showed a 7-fold increase in lung GS protein with no change in GS mRNA, indicating that an increase in GS is achieved by protein stabilization. Rats that were fed a glutamine-free diet had no changes in GS protein or mRNA levels, implying that the dynamic range of glutamine production via GS is sufficient to maintain a threshold glutamine level that keeps GS protein levels repressed (Labow et al., 1998). Taken together, these data indicate that acute physiological stress and glucocorticoid treatment

increases the amount of GS mRNA, however, a feedback mechanism that destabilizes GS protein in the presence of excess free glutamine functions to modulate the levels of GS protein expression. This system results in an increased capacity for GS expression, with an overriding post-translational regulation mechanism for the enzyme.

Intracellular aspartate is synthesized in lymphocytes via transamination of oxaloacetate by aspartate aminotransferase (AAT). AAT exists as two isozymes, cytosolic and mitochondrial, and although in most cells AAT functions as a part of the malate-aspartate "shuttle", there is a tissue-specific function of AAT in liver that is involved in increasing the supply of oxaloacetate for gluconeogenesis (Christen et al., 1985). In lymphocytes, 70% of glutamine that is metabolized beyond glutamate can be accounted for as aspartate, thus, in these cells, AAT is thought to be active in the utilization of glutamine as a metabolic fuel (Newsholme et al., 1985).

Although AAT is not subject to allosteric control, the mRNA and activity of liver AAT has been reported to be induced by glucocorticoids, with no effect on the kidney, heart, skeletal muscle, or brain enzyme. This response is enhanced by cyclic ATP (cAMP) and inhibited by insulin, with no effect on basal expression (Aggerbeck et al., 1993; Feilleux-Duche et al., 1994). The effect of these hormones is thought to be effective in stimulating the activity of this enzyme after a protein-rich meal, or after starvation (Horio et al., 1988). Promoter analysis of AAT in FAO cells revealed that the upstream region has characteristics of a housekeeping gene (absence of a TATA-box, high G + C content, multiple Sp1 sites), with an additional two glucocorticoid responsive elements (GREs). The positive and negative regulatory elements of this gene are discrete, with the cAMP element localized to -682/-26 and the negatively-regulated

insulin responsive element consisting of sequence from -1983/-1718 (Aggerbeck et al., 1993). Recently, AAT has been identified as a member of the glucose regulated protein (GRP) family, based on the induction of AAT mRNA in 3T3-F442A adipocytes cultured in glucose-free medium for 24 h (Plee-Gautier et al., 1998).

Structurally, AAT consists of a dimer of two subunits, each with a covalently linked pyridoxal 5'-phosphate coenzyme, which is essential for enzyme catalysis (Banks et al., 1968). The enzyme may be inhibited by aminooxyacetate, however, this compound has been shown to act non-specifically, inhibiting all transaminases in the plant, as well as the cytosolic and mitochondrial forms of AAT (Miflin and Lea, 1977; Meijer et al., 1975).

In summary, ASNase treatment results in the selection of drug-resistant cells that express an elevated level of AS. To supply increased aspartate and glutamine substrate to AS, resistant cells, it is my hypothesis that cells may increase amino acid transport, intracellular synthesis, or both, to maintain efficient asparagine synthesis. The source of aspartate and glutamine for AS and the resulting impact upon ASNase resistance has not, as of yet, been investigated. Thus, the purpose of these experiments is to investigate the relative contribution of extracellular versus intracellular sources of glutamine and aspartate, and to determine the impact of AS substrate availability on ASNase resistance in MOLT-4 leukemia cells.

Results

Asparagine Synthetase Substrate Transport

The active transport of aspartate by mammalian cells is primarily mediated by System $X^-_{A,G}$. However, in several independent experiments, MOLT-4 parental cells,

ASNase resistant cells, and resistant cells cultured in the absence of ASNase for 12 h (12 h-ASNase) exhibited no detectable sodium-dependent or saturable sodium-independent aspartate transport (data not shown). Also, Northern analysis revealed no measurable mRNA content for any of the isoforms (EAAT1-4) likely to encode System $X^-_{A,G}$ activity in lymphocytes. These data indicate that the development of resistance in MOLT-4 leukemia cells does not require extracellular aspartate to provide increased substrate for the AS reaction, and that ASNase treatment does not induce aspartate transport. Furthermore, since no System $X^-_{A,G}$ was present, even at a basal level, MOLT-4 lymphocytes most likely derive all their aspartate via intracellular synthesis.

In terms of glutamine uptake, MOLT-4 cells exhibited a readily measurable rate of transport activity that was significantly enhanced when ASNase resistant cells were compared to the parental MOLT-4 cell line (Figure 4-1A). Glutamine transport measured in the presence of a saturating concentration (5 mM) of the specific System A substrate MeAIB revealed that flux via this Na^+-dependent zwitterionic transport system was enhanced approximately 4-fold in the drug resistant cells. Incubation of the resistant cells in the absence of ASNase for 12 h resulted in a partial reversal of this enhanced System A activity (Figure 4-1B), indicating that the induction is largely a transient response, however, the System A transport activity of resistant cells after 12 h of ASNase withdrawal was not reduced to a level similar to that of parental cells, indicating that either there is a component of constitutive System A up-regulation in resistant cells, or the 12 h time period is not sufficient for transporter protein to be completely down-regulated.

To determine the rate of glutamine uptake via System ASC in MOLT-4 cells, transport was performed in the presence of a saturating concentration of unlabeled serine (5 mM) as well as MeAIB, to eliminate flux via System A. These data indicate that the Na^+-independent uptake of glutamine is minimal and that the balance of Na^+-dependent uptake in MOLT-4 cells is primarily mediated by System ASC (Figure 4-1A). Overall, the contribution of System ASC to glutamine uptake in the parental cells is approximately 3-fold that of System A, consistent with studies indicating that System ASC is the primary zwitterionic transport agency in lymphocytes (Schhroder, 1990).

Analogous to System A, the ASC activity was enhanced more than 4-fold in ASNase resistant cells cultured continuously in ASNase, however, after 12 h of ASNase withdrawal from resistant cells, the System ASC activity returned almost completely to the rate observed in parental cells (Figure 4-1). These data indicate that the adaptive response of ASC transport induced by ASNase treatment is reversible within a period of 12 h, with no component of long-term constitutive up-regulation.

To examine the transcriptional regulation of System ASC, Northern analysis was performed on parental and resistant MOLT-4 cells using the cDNA clones available for the two genes that encode ASC transport activity (Arriza et al., 1993; Utsonomiya-Tate et al., 1996). Although Northern analysis of poly $(A)^+$ mRNA indicated little or no ASCT2 expression (data not shown), ASCT1 mRNA was detectable as three species; 2.2, 3.5, and 5.0 Kb (Figure 4-2) in length, consistent with the ASCT1 mRNAs observed in other cell types (Arriza et al., 1993; Shafquat et al., 1993). Although the quantitation of the 3.5 Kb band is presented in Figure 4-2, densitometry measurements not shown verified that the relative expression of the 5.0 and 2.2 Kb bands were identical to the 3.5 Kb transcript.

In parallel to the System ASC activity measurements, the ASCT1 mRNA content was increased in response to ASNase exposure for both parental and resistant MOLT-4 cells (Figure 4-2, lanes 2 and 4). Also, the ASCT1 mRNA in resistant cells cultured in the absence of ASNase for 12 h was reduced to parental control levels, indicating that the ASCT1 mRNA was completely down-regulated by this time. Resistant cells that had been maintained in medium lacking ASNase for 6 weeks (6wk-ASNase) also express a basal level of ASCT1 mRNA (Figure 4-2, lane 5) equal to parental control levels, indicating that there is no long-term constitutive up-regulation of ASCT1 mRNA levels in resistant cells. Taken together, the data illustrate that both ASC activity and ASCT1 mRNA are increased in response to ASNase treatment of MOLT-4 cells, and that down-regulation to parental control levels following drug removal occurs rapidly. The slight lag in decay of transporter activity (Figure 4-1B) compared to the ASCT1 mRNA (Figure 4-2) may indicate that the ASC protein is not completely down-regulated after ASNase withdrawal, perhaps owing to a long half-life of the ASCT1 protein.

The Northern blot was also probed with a cDNA corresponding to the 3' coding region of human System A (Sugawara et al., 2000), and a similar pattern of transcriptional regulation was identified for this transporter. The level of System A mRNA was increased approximately 25% upon exposure to ASNase for 12 h for parental and resistant cells (Figure 4-3, lanes 2 and 4), and in resistant cells, the enhanced System A mRNA returns to the basal level found in parental control cells after a 12 h incubation in complete medium (Figure 4-3, lane 4). Also, the System A mRNA is not constitutively up-regulated in resistant cells, since resistant cells maintained in the absence of ASNase for 6 weeks express a basal level of System A mRNA equal to that of

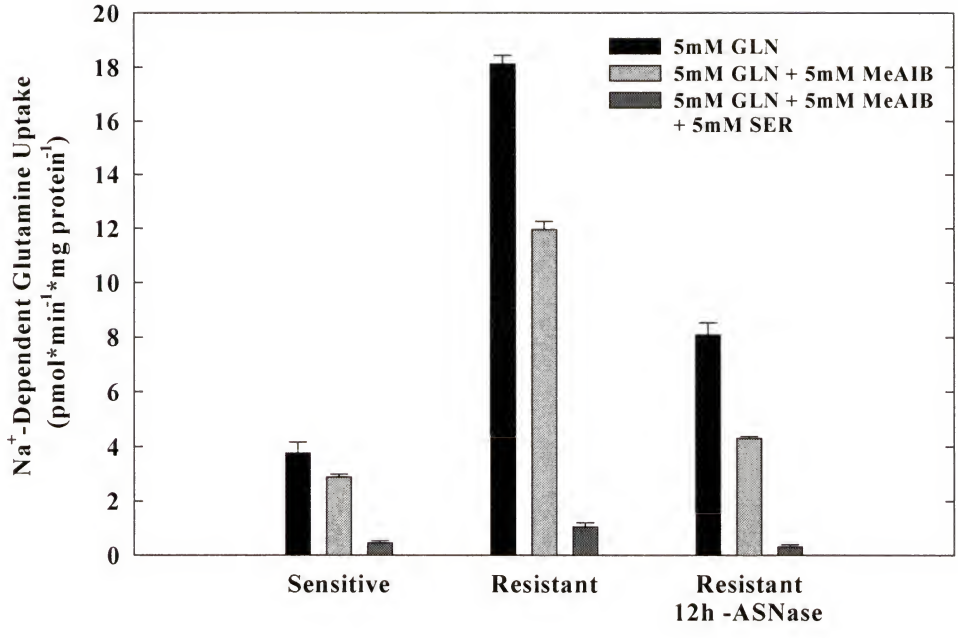
parental control cells (Figure 4-3, lane 5). Taken together, these data indicate that the transcriptional regulation of System A in response to ASNase treatment and selection is similar to that of System ASC in that the transporter mRNA is enhanced upon ASNase treatment, however, the mRNA decays quickly after ASNase withdrawal, and there is no component of long-term constitutive up-regulation.

In addition to monitoring the changes in uptake for the AS substrates aspartate and glutamine, it was also of interest to determine whether amino acid efflux across the plasma membrane may be altered in drug-resistant cells. The primary Na^+-independent activity that would permit efflux from mammalian cells is System L. This bi-directional transport activity is monitored by measuring the Na^+-independent uptake of leucine inhibited by a saturating concentration of BCH, a non-metabolizable, System L-specific amino acid analog. The data illustrate that resistant cells cultured in the presence of ASNase have reduced System L activity relative to the parental cells (Figure 4-4), and that withdrawal of ASNase for 12 h resulted in a complete normalization of the System L, indicating that the reduction in activity is a transient adaptation to amino acid availability, similar to that observed for System ASC.

Collectively, the transport data document that the uptake of glutamine is enhanced in ASNase-resistant MOLT-4 cells as compared to parental cells, whereas little or no active transport for aspartate is observed in either cell population. Also, the primary activity responsible for amino acid efflux, System L, is actually down-regulated in ASNase resistant cells, potentially serving to reduce the exodus of substrate amino acids during ASNase treatment. The adaptive responses of both Systems ASC and L were

Figure 4-1. Glutamine Transport in MOLT-4 Lymphocytes. To determine the rates of glutamine transport in parental and resistant MOLT-4 lymphocytes, cells were subjected to an amino acid transport assay as described in Chapter 2. To identify the individual transport systems mediating Na^+-dependent glutamine uptake, a saturating concentration (5mM) of unlabeled MeAIB was included to competitively inhibit transport via System A. The inclusion of saturating (5mM) unlabeled serine additionally inhibited transport via System ASC. To examine the reversibility of ASNase effects on transport, resistant MOLT-4 cells were incubated in medium lacking ASNase for 12h prior to transport (A). The subtracted velocities for the individual Systems A and ASC have been computed and graphed in (B). The data presented are the averages ± standard deviations (SD) of quadruplicate samples and are representative of three independent experiments.

A


Na+-Dependent Glutamine Uptake
(pmol*min-1*mg protein-1)
20
18
16
14
12
10
8
6
4
2
0
5mM GLN
5mM GLN + 5mM MeAIB
5mM GLN + 5mM MeAIB
+ 5mM SER
Sensitive
Resistant
Resistant
12h -ASNase

B

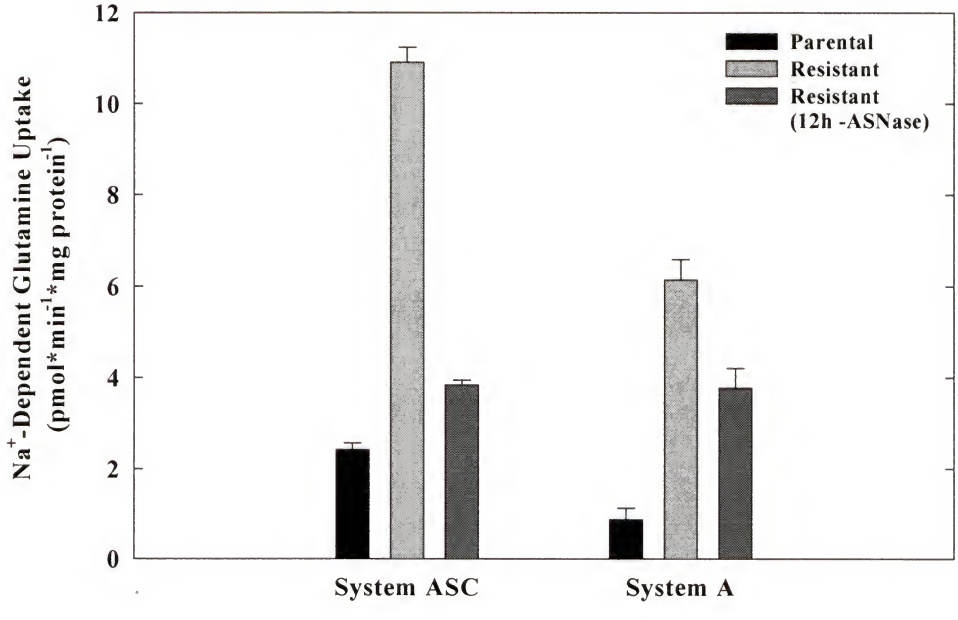

Na+-Dependent Glutamine Uptake
(pmol*min-1*mg protein-1)
12
10
8
6
4
2
0
Parental
Resistant
Resistant
(12h -ASNase)
System ASC
System A

Figure 4-2. ASCT1 mRNA Expression in MOLT-4 Lymphocytes. To examine the induction of mRNA encoding System ASC as a result of ASNase treatment, parental and resistant MOLT-4 cells (1×10^4 cells/mL) were incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase for 12h and subjected to northern analysis as described in Chapter 2. The reversibility of ASCT1 mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human ASCT1, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental control cells and plotted as a bar graph. The data presented are representative of two independent experiments.

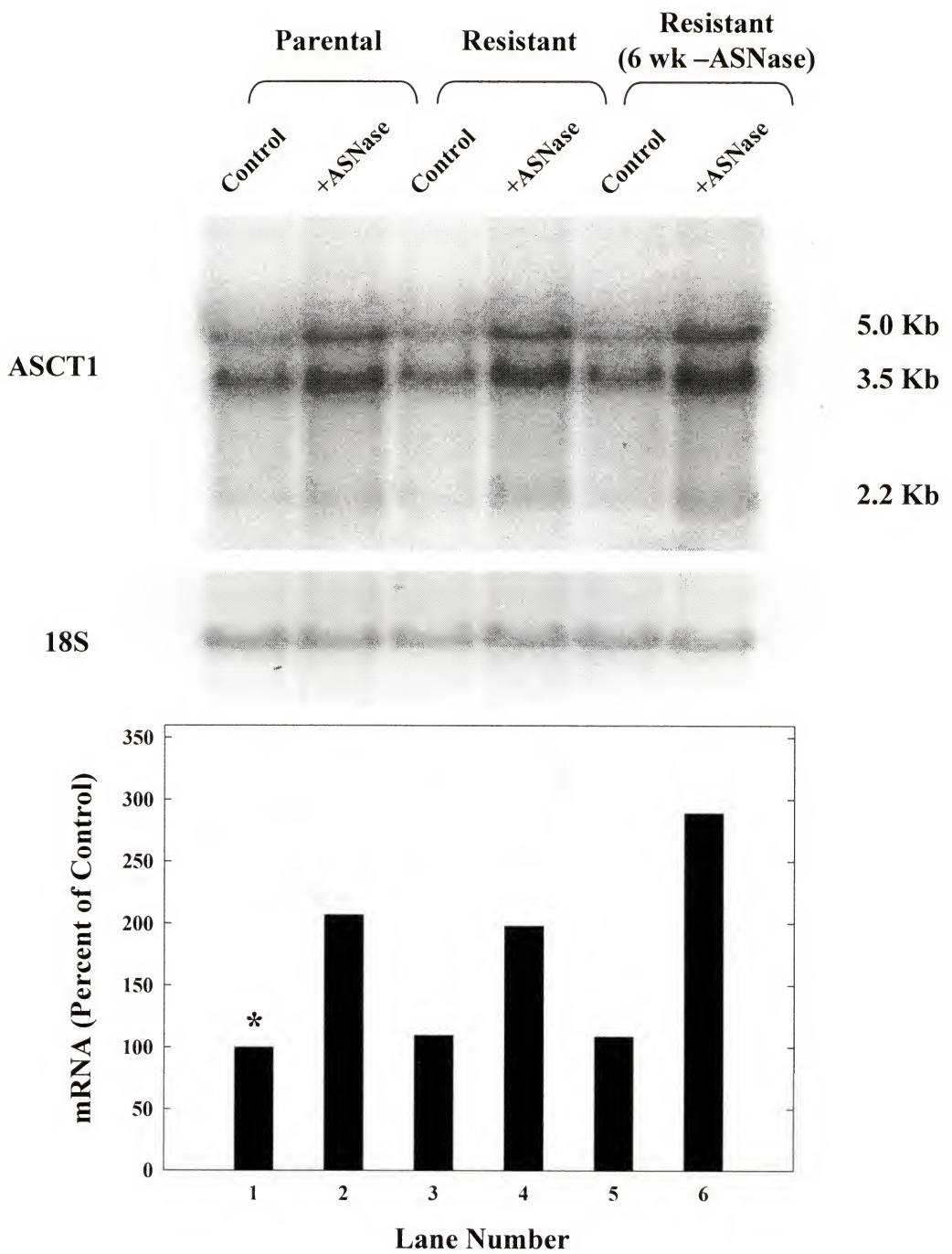

Parental
Resistant
Resistant
(6 wk –ASNase)
Control
+ASNase
Control
+ASNase
Control
+ASNase
ASCT1
5.0 Kb
3.5 Kb
2.2 Kb
18S
mRNA (Percent of Control)
350
300
250
200
150
100
50
0
*
1
2
3
4
5
6
Lane Number

Figure 4-3. System A mRNA Expression in MOLT-4 Lymphocytes. To examine the induction of mRNA encoding System A as a result of ASNase treatment, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase for 12h and subjected to northern analysis as described in Chapter 2. The reversibility of System A mRNA regulation was determined by incubating resistant cells in the absence of ASNase for 6 weeks before analysis. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the 3' coding region of human System A, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental control cells and plotted as a bar graph. The data presented are representative of two independent experiments.

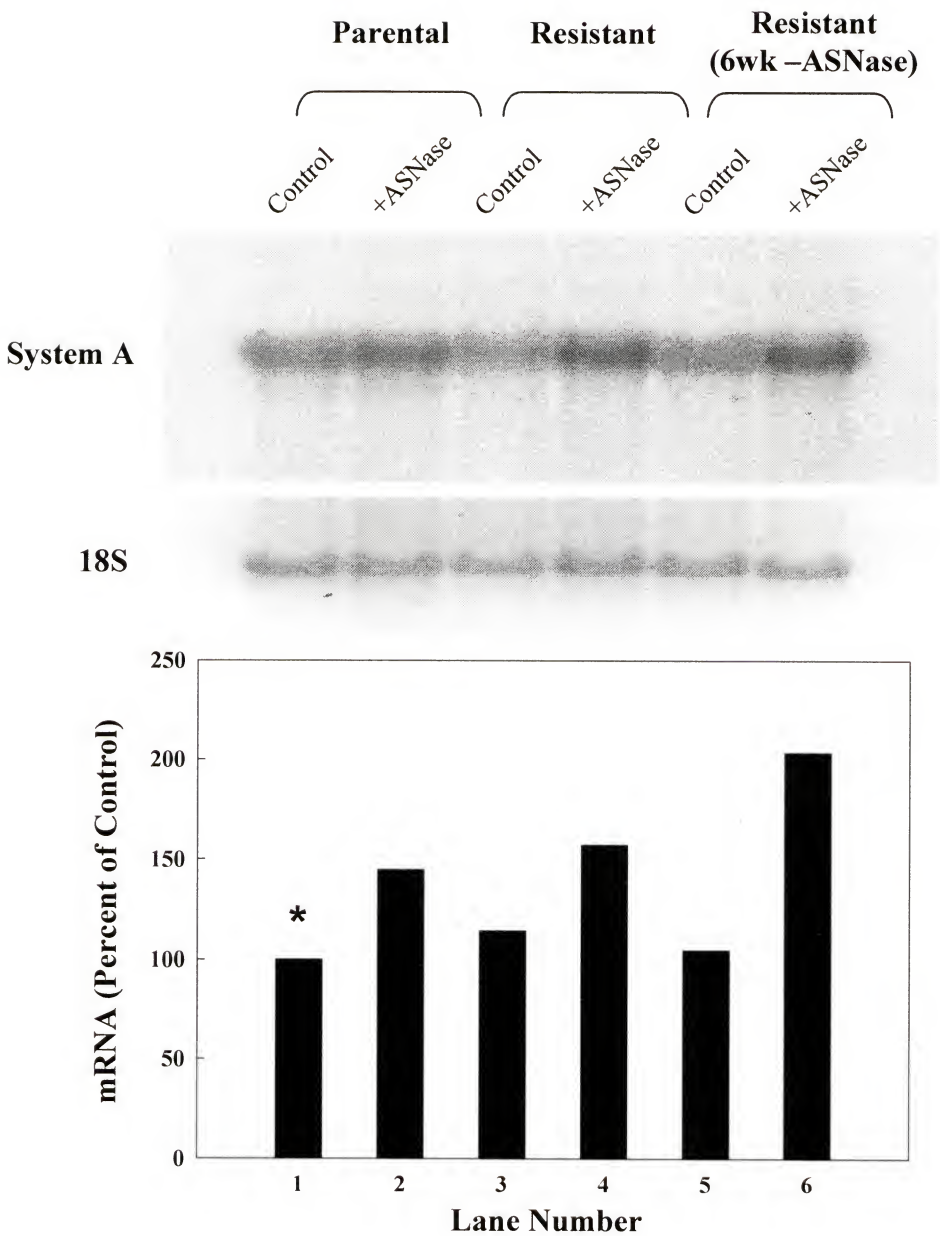
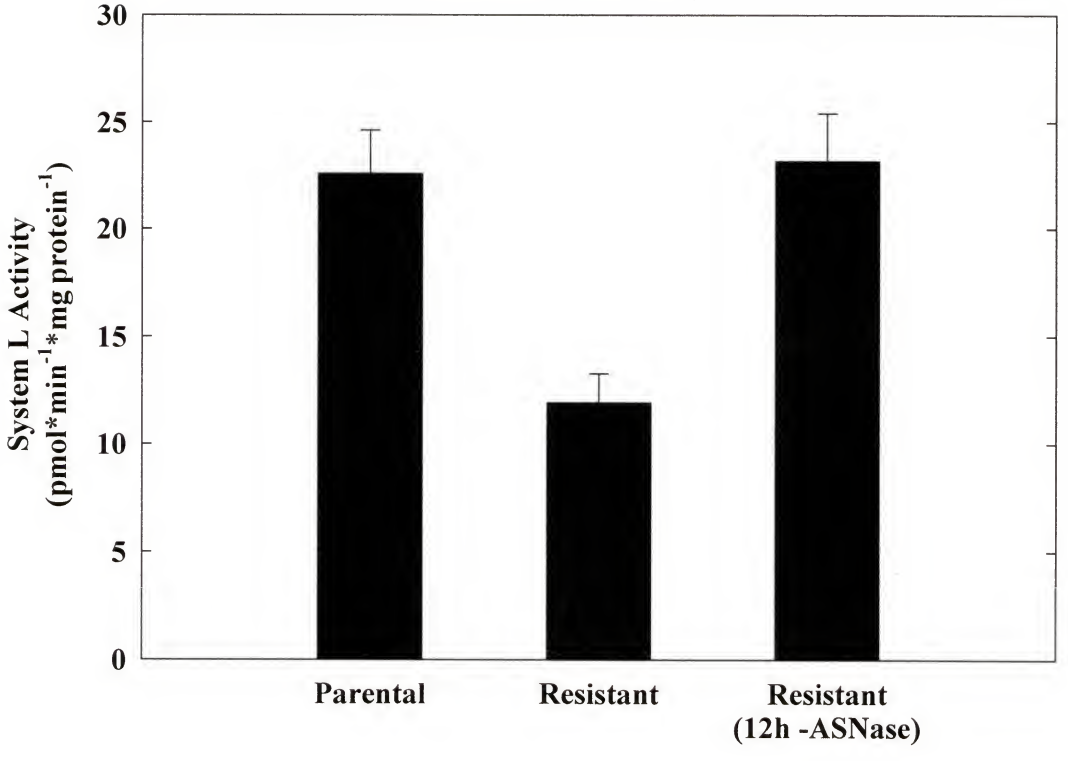

Parental
Resistant
Resistant
(6wk –ASNase)
Control
+ASNase
Control
+ASNase
Control
+ASNase
System A
18S
mRNA (Percent of Control)
250
200
150
100
50
0
*
1
2
3
4
5
6
Lane Number

Figure 4-4. Activity of System L in MOLT-4 Lymphocytes. The activity of System L, a Na^+- independent amino acid transport system, was assayed in parental and resistant cells by measuring the uptake of leucine in the absence of Na^+ and in the presence of a saturating concentration (5mM) of the specific System L inhibitor, 2-amino-2-norbornanecarboxcylic acid (BCH) as described in Chapter 2. The reversibility of System L was monitored by incubation of resistant cells in medium lacking ASNase for 12h prior to transport. The data presented are the averages ± standard deviations (SD) of quadruplicate samples and are representative of three independent experiments.


30
25
20
15
10
5
0
System L Activity
(pmol*min-1*mg protein-1)
Parental
Resistant
Resistant
(12h -ASNase)

rapidly reversible, whereas the enhanced activity of System A in resistant cells was reversed less effectively after 12 h of ASNase withdrawal, indicating a potential long-term constitutive enhancement in these cells. Trans-effects due to changes in cytoplasmic amino acid pools were eliminated as a factor in these experiments by incubating the cells in amino acid-free medium for 30 min prior to the transport assays. Therefore, the data suggest that ASNase treatment causes transient changes in amino acid transporter expression that augment the capacity of the cell to concentrate the AS substrate glutamine.

System A and ASNase Resistance

Increased System A transport activity has been correlated with the cell cycle as well as increased cell growth (Saier et al., 1988; Kilberg and Handlogden, 1988). System A induction is the permissive step in liver regeneration, and the blockade of System A activity by the specific substrate MeAIB results in suppression of DNA synthesis and liver regeneration *in vivo*, as well as inhibition of hepatocyte proliferation *in vitro*. (Leffert et al., 1988; Freeman et al., 1999). Given that System A is up-regulated in resistant MOLT-4 leukemia cells, it was of interest to determine whether this heightened activity was essential for growth in the presence of ASNase.

To investigate the role of elevated System A activity in maintenance of ASNase resistance, parental and ASNase-resistant MOLT-4 cells were incubated for 48 h in medium containing 0-10 mM MeAIB. After incubation, the growth rates of the two cell populations were determined by measuring the number of viable cells using the WST-1 assay (Figure 4-5A). The inclusion of MeAIB had no significant effect on cell growth of the parental MOLT-4 cells, however, incubation of ASNase resistant cells in MeAIB

resulted in a concentration-dependent decline in cell growth relative to resistant cells incubated in the absence of the amino acid analog (Figure 4-5A). In a separate experiment not presented, it was determined that 5 mM MeAIB completely inhibited flux via System A in MOLT-4 cells. Interestingly, there was no reduction in cell growth in MOLT-4 cells cultured in 10 mM MeAIB as compared to 5 mM MeAIB, consistent with the observation that 5 mM MeAIB is sufficient to saturate this carrier. The data indicate that amino acid transport mediated by System A is an important contributor to cell growth of drug resistant MOLT-4 cells maintained in the presence of ASNase.

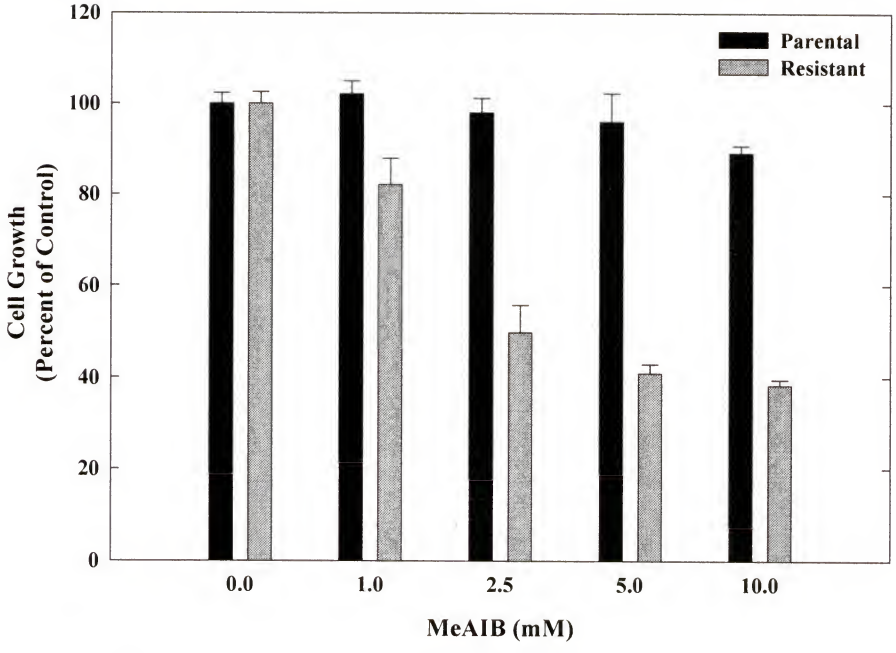
Consistent with these observations, a 48 h incubation of parental cells in the presence of 5 mM MeAIB caused no significant change in total cell viability (96.4% vs. 98.4%) or the number of cells undergoing apoptosis (1.3% vs 0.4%) (Figure 4-5B). For resistant cells continuously cultured in the presence of ASNase, however, MeAIB treatment induced a small, but detectable decrease in the percentage of viable cells (96.6% vs. 90.5%) and an increase in apoptotic cells (1.0% vs 7.4%). These data confirm that amino acid transport mediated by System A is required for viability and growth of ASNase resistant cells.

Aspartic Acid Metabolism

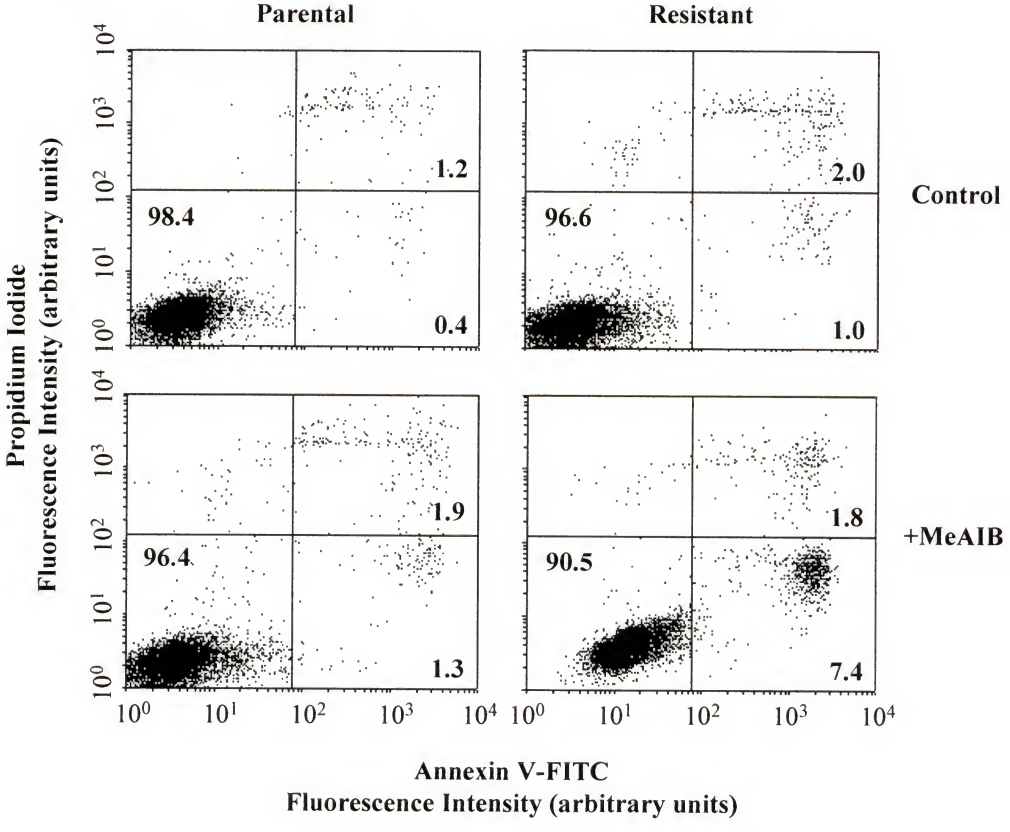
Given that neither parental nor ASNase resistant MOLT-4 cells express detectable levels of aspartate transport, the availability of this AS substrate must depend on intracellular synthesis by transamination of oxaloacetate by aspartate aminotransferase (AAT). Unfortunately, there is no specific inhibitor for AAT, so the only method for investigating the impact of aspartate deprivation was to incubate parental and resistant

Figure 4-5. Effect of MeAIB on Cell Growth and Viability. To determine the effect of increased System A transport on cell growth and viability, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated for 48h in the absence (parental) or presence (resistant) of 1U/mL ASNase. To competitively inhibit transport via System A, the indicated concentrations of MeAIB were included in the medium. The cells were then subjected to a WST-1 cell proliferation assay (A) and an annexin V-FITC apoptosis assay (B) as described in Chapter 2. For the WST-1 cell growth assay (A), the absorbance of the treated cells is plotted as a percentage (± SD, n=4) of the untreated control for each individual cell population. For the apoptosis assay (B), the numbers in each quadrant indicate the percentage of cells from a total of 10,000 counted. The data presented are representative of three independent experiments.

A


Parental
Resistant
Cell Growth
(Percent of Control)
120
100
80
60
40
20
0
0.0
1.0
2.5
5.0
10.0
MeAIB (mM)

B


Parental
Resistant
Propidium Iodide
Fluorescence Intensity (arbitrary units)
98.4
1.2
0.4
96.6
2.0
1.0
Control
96.4
1.9
1.3
90.5
1.8
7.4
+MeAIB
Annexin V-FITC
Fluorescence Intensity (arbitrary units)

MOLT-4 cells in the presence of the general transaminase inhibitor, aminooxyacetate (AOA). Inhibition of general transamination caused a 70-80% decrease in the growth of both sensitive and resistant cells as monitored by the WST-1 assay (Figure 4-6). In fact, AOA treatment was nearly as cytostatic as ASNase treatment for the parental cells, indicating the importance of intracellular transamination for the proliferation of both cell populations. Accordingly, when parental cells were treated with AOA and subjected to an annexin V apoptosis assay (Figure 4-7), there was an AOA-induced apoptosis and subsequent cell death that was further magnified by the simultaneous addition of ASNase. In contrast, the addition of AOA alone did not cause significant apoptosis in the ASNase resistant cell population, however, some cell death did occur during ASNase treatment. These data indicate that for the ASNase resistant cells, the decrease in total cell number as measured by the WST-1 assay is primarily due to a cytostatic action on the treated cells relative to the non-treated cells. Also, the relatively minor effect of AOA on apoptosis in the ASNase resistant cells, with or without ASNase for the 48 h of the incubation, documents that these cells have undergone an adaptation that makes them less dependent on amino acid transamination for cell survival.

Glutamine Metabolism

E. coli ASNase contains a glutaminase side reaction that rapidly hydrolyzes medium glutamine *in vitro* and plasma glutamine *in vivo*, possibly contributing to its effectiveness as a neoplastic agent. In fact, the reduction of extracellular glutamine may affect the ability of the cell to synthesize asparagine during ASNase treatment, because the extracellular source of this amino acid is essential for macromolecular synthesis and energy production in lymphocytes.

Figure 4-6. Effect of Transamination Inhibition on Cell Growth in MOLT-4 Cells. To determine the contribution of transamination to cell growth and ASNase resistance, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated for 48h in the presence of the general transaminase inhibitor, AOA with (+ASNase), or without (control) 1U/mL ASNase and subjected to a WST-1 cell growth assay as described in Chapter 2. The absorbance of the AOA-treated cells is plotted as a percentage (± SD, n=4) of the untreated control for each individual cell population. The data presented are representative of three independent experiments.

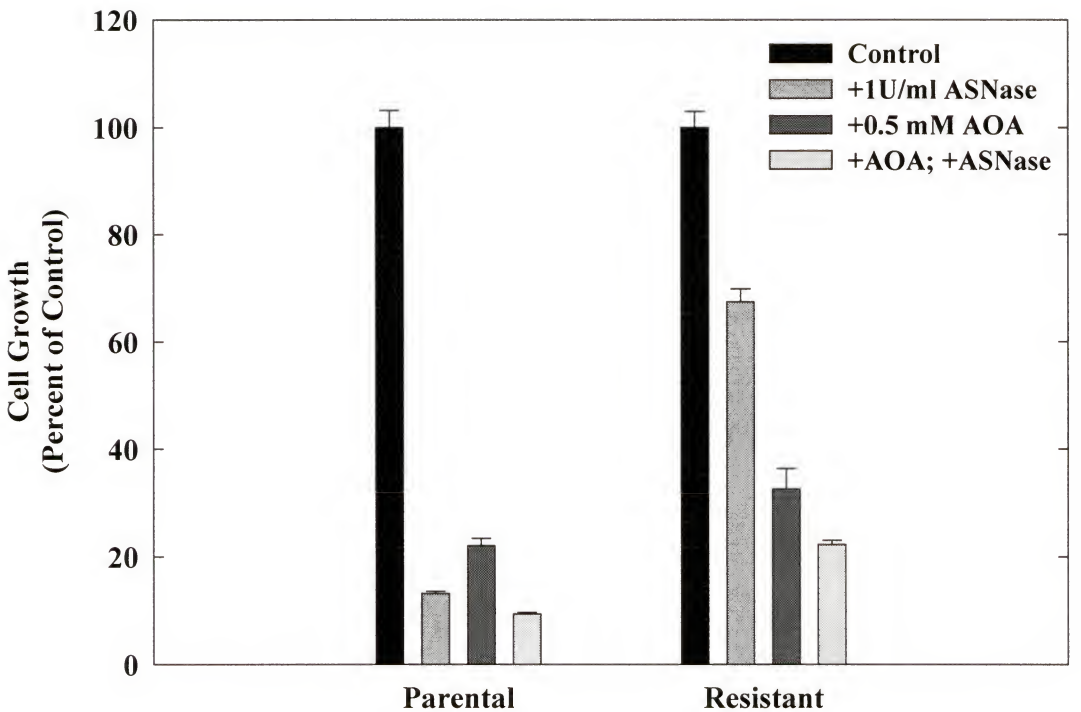
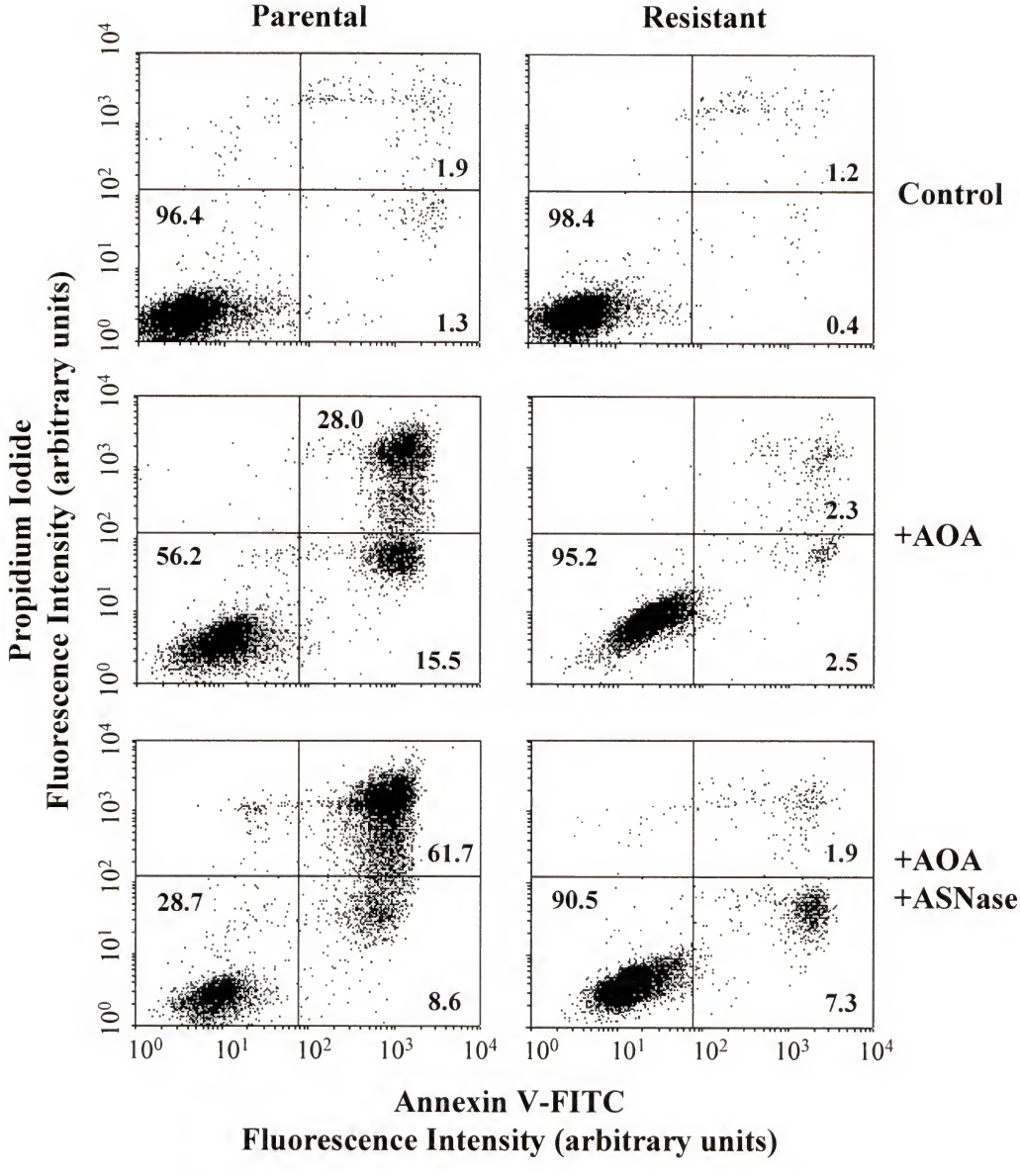

120
100
80
60
40
20
0
Cell Growth
(Percent of Control)
Control
+1U/ml ASNase
+0.5 mM AOA
+AOA; +ASNase
Parental
Resistant

Figure 4-7. Effect of Transamination Inhibition on Apoptosis in MOLT-4 Cells. To determine the contribution of transamination to cell viability and ASNase resistance, parental and resistant MOLT-4 cells (1 x 10^4 cells/mL) were incubated for 48h in the presence of the transaminase inhibitor AOA with (+ASNase), or without (control) 1U/mL ASNase and subjected to an annexin V-FITC apoptosis assay as described in Chapter 2. The numbers in each quadrant indicate the percentage of cells from a total of 10,000 counted. The data presented are representative of three independent experiments.


Parental
Resistant
Control
+AOA
+AOA
+ASNase
1.9
96.4
1.3
1.2
98.4
0.4
28.0
56.2
15.5
2.3
95.2
2.5
61.7
28.7
8.6
1.9
90.5
7.3
Propidium Iodide
Fluorescence Intensity (arbitrary units)
Annexin V-FITC
Fluorescence Intensity (arbitrary units)

To document the importance of extracellular glutamine availability in MOLT-4 lymphocytes, parental and resistant cells were cultured for 48 h in decreasing concentrations of medium glutamine in the absence or presence of the GS inhibitor, MSO. Incubation of the parental cells in decreasing extracellular glutamine (Figure 4-8A) resulted in a concentration-dependent reduction of cell proliferation, indicating that extracellular glutamine is necessary to maintain cell growth. Inhibition of glutamine synthesis by MSO had little effect at extracellular glutamine concentrations above 0.5 mM, but MSO-dependent decreases in cell growth were observed as the medium glutamine concentration was decreased from 0.5 to 0 mM (Figure 4-8A). For example, the number of viable cells in glutamine-free medium was approximately 55% of control in the absence of MSO after the 48 h incubation, but less than 10% of control in the presence of the GS inhibitor. Given that plasma glutamine content is approximately 0.5-0.7 mM, these data indicate that extracellular glutamine is the primary source of this amino acid and that glutamine synthesis impacts parental MOLT-4 cell growth only when extracellular glutamine becomes limiting, as would be the case during ASNase treatment. In addition, the decline in cell proliferation in the absence of glutamine indicates that the basal glutamine synthetic rate is not sufficient to maintain optimal growth.

When ASNase-resistant MOLT-4 cells were incubated in the absence of ASNase and in decreasing medium glutamine, cell proliferation was reduced and further reduction also occurred in the presence of MSO (Figure 4-8B). In the presence of ASNase, MSO modestly potentiated the growth-inhibitory effect of the drug on the resistant cells. These data indicate that ASNase resistant cells rely heavily on extracellular glutamine for

optimal growth, and that in the presence of ASNase, intracellular glutamine synthesis is not sufficient to maintain optimal growth.

In terms of cell death during glutamine deprivation, when parental MOLT-4 cells were incubated for 48 h in 0, 0.1, or 2 mM glutamine, cell viability declined by approximately 10% at the two sub-physiologic concentrations with a concurrent increase in the number of cells undergoing apoptosis and/or necrosis (Table 4-1). The presence of 0.5 mM MSO had little effect on cell viability in the presence of 2 mM glutamine, but significantly decreased cell viability was observed when extracellular glutamine became limiting. Taken together, the data indicate that in the absence of extracellular glutamine, MOLT-4 cells are primarily cytostatic, however, when intracellular glutamine synthesis is inhibited, the parental cells undergo significant apoptosis during glutamine deprivation.

As expected, treatment of the parental cells with ASNase resulted in a 60-65% decline in cell viability indicating, along with the data of Figure 3-1B, that ASNase treatment is both cytostatic and cytotoxic. After the 48 h time period investigated, 16-18% of the ASNase treated cells were apoptotic, whereas 42-44% were necrotic (Table 4-1). When the parental cells were treated with both ASNase and the GS inhibitor MSO, there was a further reduction in cell viability beyond that seen in the presence of ASNase alone.

The drug-resistant MOLT-4 cells were relatively similar to the parental cells in their response to limitation of extracellular glutamine or blockade of intracellular synthesis when the WST-1 assay was used to monitor the growth effects of these treatments. In contrast, when apoptosis was monitored, significant differences were revealed between the parental and drug resistant cell populations. Limiting the

extracellular glutamine concentration resulted in no significant decline in cell viability (Table 4-1) despite the cytostatic effect (Figure 4-8). Furthermore, inhibition of glutamine synthesis at extracellular concentrations of 0 or 0.1 mM glutamine resulted in a severe decrease in cell growth (Figure 4-8), however, the viability of these cells was not substantially affected. In fact, when both extracellular and intracellular glutamine sources were minimized, greater than 80% of the ASNase resistant cells remained viable for the 48 h period of treatment (Table 4-1). These data provide a striking contrast between parental and ASNase resistant MOLT-4 cells and indicate that the drug resistant cells are much less dependent on glutamine for cell survival. Similar results were obtained whether or not ASNase was included in the incubations (Table 4-1, compare plus MSO versus plus ASNase plus MSO).

Kiriyama et al (1989) documented that GS levels of ASNase resistant U937 cells were increased by 30% as compared to cells selected in medium prepared without asparagine, indicating that ASNase treatment, which depletes both glutamine and asparagine, may select for a glutamine-independent phenotype that includes enhanced GS activity. To establish whether or not ASNase treatment results in activation of GS expression in MOLT-4 cells, the GS mRNA content and activity was monitored in both parental and ASNase resistant cells cultured in the absence or presence of the drug. Incubation of parental MOLT-4 cells in ASNase for 12 h caused little or no change in GS mRNA content (Figure 4-9, compare lanes 1 and 2). ASNase resistant cells exhibited a slightly elevated level (~20%) of GS mRNA (Figure 4-9, lane 4), relative to the parental cells that did not decline when they were incubated for only 12 h without the drug (Figure 4-9, lane 3). Culture of ASNase resistant cells in the absence of drug for 6 weeks (Figure

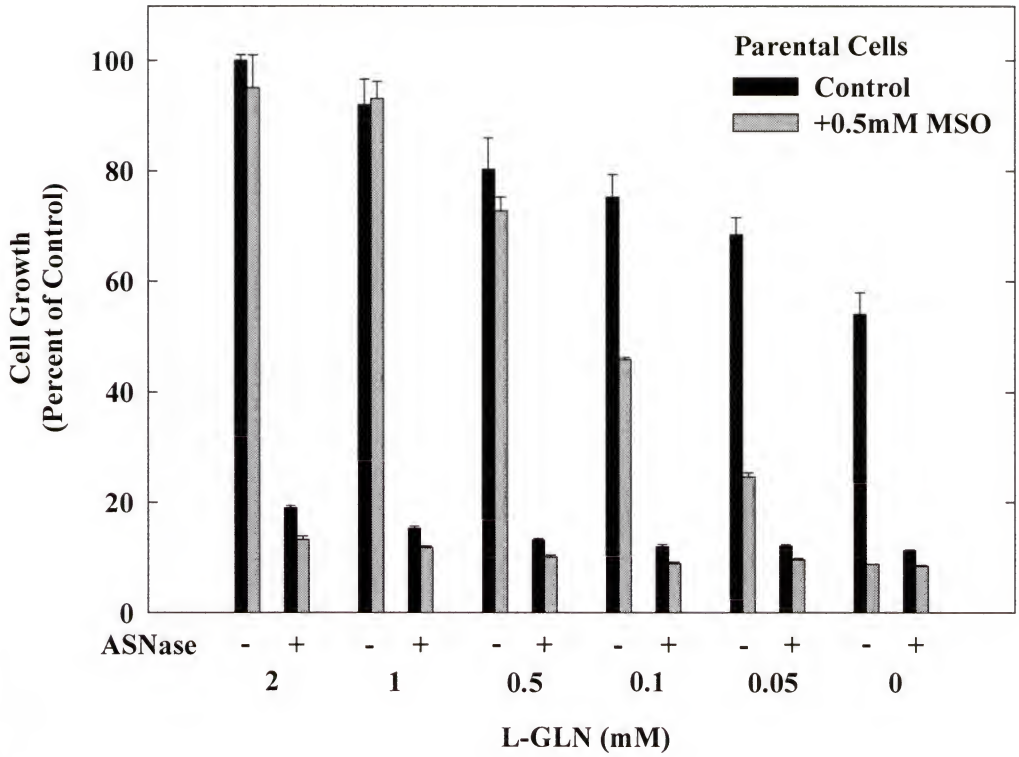
4-9, lane 5) also did not result in a return of the elevated GS mRNA content to the level present in parental cells (Figure 4-9, lane 1). Collectively, these data document that the relative abundance of GS mRNA in MOLT-4 cells is not enhanced by short-term (12 h) decreases in asparagine or glutamine availability, but exhibits a modest level of long-term adaptation in ASNase resistant cells that appears to be relatively stable in terms of reversibility.

It is known that GS enzymatic activity is subject to regulation by post-transcriptional and post-translational mechanisms (Lie-Venema et al., 1988, Meister et al., 1974), therefore, enzyme activity was monitored in parental and drug resistant MOLT-4 cells incubated in the absence or presence of ASNase for 12 h (Figure 4-10). Incubation of parental cells in the presence of ASNase for 12 h caused a significant increase in GS enzymatic activity (23±4 versus 99±12 pmol•mg^{-1}protein•min^{-1}). Also, resistant cells continuously cultured in the presence of the drug exhibited a high level of GS activity, approximately 4-fold that of parental cells exposed to ASNase for 12 h. Considering the intracellular amino acid analysis in table 3-1, a reduction of the intracellular glutamine levels in ASNase treated cells may result in the activation of GS, however, it is clear that resistant cells have a higher capacity for GS up-regulation in response to ASNase exposure than parental cells.

The data also illustrate that short-term (12 h) removal of the drug from the resistant cells resulted in a large decline in GS activity (425±8 versus 113±8 pmol•mg^{-1}protein•min^{-1}), perhaps because of substrate destabilization of the enzyme. Collectively, the data are consistent with previous reports documenting post-transcriptional control as the primary mechanism for regulation of GS activity

Figure 4-8. Effect of Glutamine Synthesis Inhibition on Cell Growth in MOLT-4 Cells. To determine the contribution of intracellular glutamine synthesis via GS to cell growth and ASNase resistance, parental (A) and resistant (B) MOLT-4 cells (1×10^4 cells/mL) were incubated for 48h in the presence of the glutamine synthesis inhibitor MSO with (+ASNase), or without (control) 1U/mL ASNase, and subjected to a WST-1 cell growth assay as described in Chapter 2. In addition, the impact of decreasing extracellular glutamine on cell growth was investigated by assaying each treatment in the presence of 0, 0.05, 0.1, 0.5, 1, and 2mM medium glutamine. The absorbance of the treated cells is plotted as a percentage (± S.D., n=4) of the untreated control for each individual cell population. The data presented are representative of three independent experiments.

A


Parental Cells
Control
+0.5mM MSO
Cell Growth
(Percent of Control)
100
80
60
40
20
0
ASNase - + - + - + - + - + - +
2 1 0.5 0.1 0.05 0
L-GLN (mM)

B

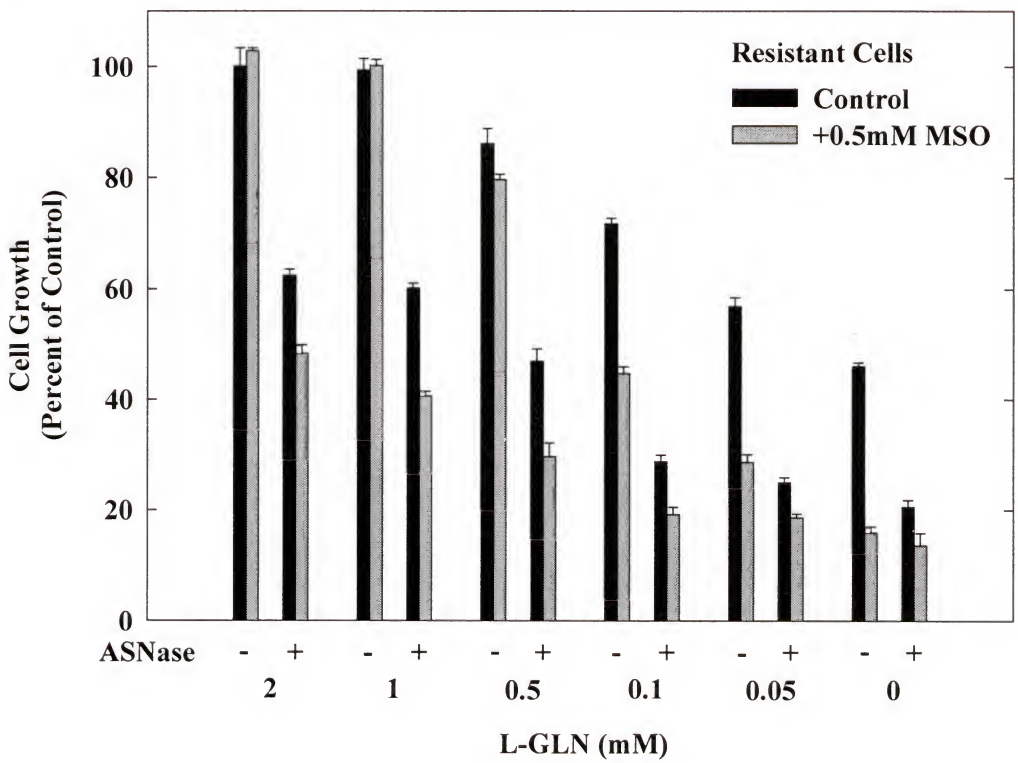

Resistant Cells
Control
+0.5mM MSO
Cell Growth
(Percent of Control)
100
80
60
40
20
0
ASNase - + - + - + - + - + - +
2 1 0.5 0.1 0.05 0
L-GLN (mM)

Table 4-1. Effect of Glutamine Synthesis Inhibition on Apoptosis in MOLT-4 Cells. To determine the contribution of intracellular glutamine synthesis via GS to cell viability and ASNase resistance, parental (A) and resistant (B) MOLT-4 cells (1×10^4 cells/mL) were incubated for 48h in the presence of the glutamine synthesis inhibitor MSO with (+ASNase), or without (control) 1U/mL ASNase, and subjected to an annexin V-FITC apoptosis assay as described in Chapter 2. In addition, the impact of decreasing extracellular glutamine on cell growth was investigated by assaying each treatment in the presence of 0, 0.1, and 2mM medium glutamine. The data presented are the percentage of cells from a total of 10,000 counted and are the averages ± standard deviations (SD) from three independent expreriments.

Apoptosis Analysis of Parental and Resistant MOLT-4 Cells After 12h of ASNase Treatment with 0.5mM MSO in 0, 0.1, and 2mM Glutamine

Parental Cells	Control			+MSO			+ASNase			+ASNase, +MSO			
	Viable	Apoptotic	Necrotic	Viable	Apoptotic	Necrotic	Viable	Apoptotic	Necrotic	Viable	Apoptotic	Necrotic	
2mM GLN	**91.44**	**4.05**	**3.80**	**87.08**	**6.62**	**5.59**	**35.38**	**18.21**	**44.11**	**33.96**	**29.84**	**34.35**	**Average**
	0.93	0.89	1.76	4.11	1.84	4.25	1.65	7.83	6.03	14.61	7.47	14.73	S.D.
0.1mM GLN	**81.46**	**7.92**	**10.07**	**48.56**	**18.67**	**31.45**	**38.48**	**16.83**	**43.72**	**27.15**	**28.37**	**42.51**	**Average**
	2.60	2.02	4.18	6.90	6.91	7.19	3.33	7.41	6.19	7.85	9.89	11.27	S.D.
0mM GLN	**80.56**	**8.20**	**10.52**	**27.82**	**26.98**	**42.97**	**40.88**	**16.03**	**42.14**	**28.47**	**25.33**	**43.34**	**Average**
	0.99	2.75	3.40	6.81	9.15	12.50	1.39	6.84	6.92	7.44	8.42	9.20	S.D.

Resistant Cells	Control			+MSO			+ASNase			+ASNase, +MSO			
2mM GLN	**96.30**	**1.10**	**1.73**	**95.91**	**1.74**	**1.82**	**91.05**	**4.01**	**4.42**	**92.62**	**3.94**	**2.70**	**Average**
	1.93	0.96	1.05	1.07	0.40	0.64	3.38	1.67	1.57	2.73	1.13	1.66	S.D.
0.1mM GLN	**92.94**	**2.65**	**3.47**	**82.26**	**8.38**	**7.30**	**78.57**	**10.05**	**10.34**	**86.53**	**7.08**	**5.03**	**Average**
	1.90	0.91	1.16	5.57	5.65	2.41	4.45	1.70	6.42	2.69	1.74	1.79	S.D.
0mM GLN	**92.32**	**3.79**	**3.24**	**83.49**	**9.72**	**5.76**	**79.07**	**7.34**	**12.00**	**83.39**	**8.70**	**6.50**	**Average**
	2.14	1.39	0.96	2.96	1.36	2.22	7.18	2.31	6.37	2.52	1.69	2.43	S.D.

Figure 4-9. Expression of Glutamine Synthetase mRNA in MOLT-4 Cells. To assess the transcriptional regulation of GS in MOLT-4 lymphocytes during ASNase treatment, parental and resistant cells (1×10^4 cells/mL) were incubated for 12h in the absence (control) or presence (+ASNase) of 1U/mL ASNase and subjected to northern analysis as described in Chapter 2. The blot (15μg/lane) was probed with [^{32}P] radiolabeled cDNAs corresponding to the coding region of human GS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental control cells and plotted as a bar graph. The data presented are the averages ± standard deviations (SD) of three independent experiments.

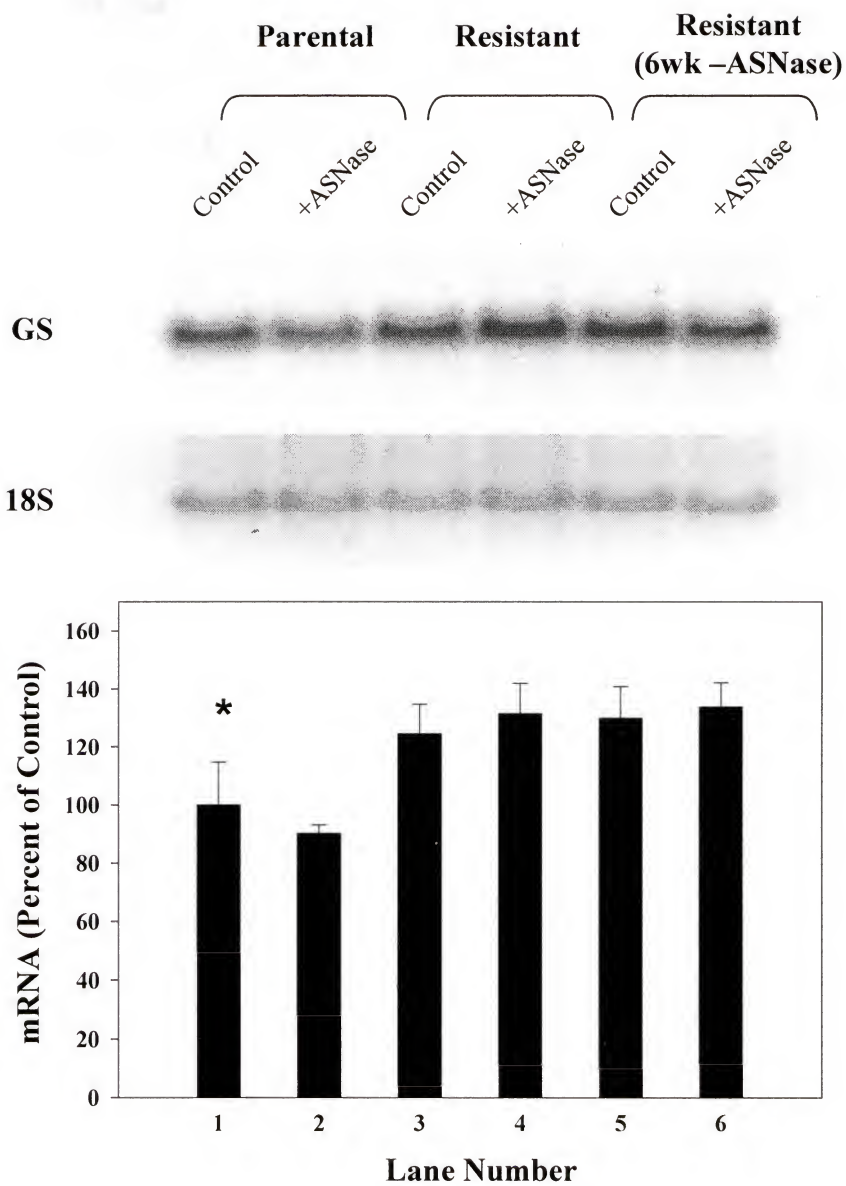
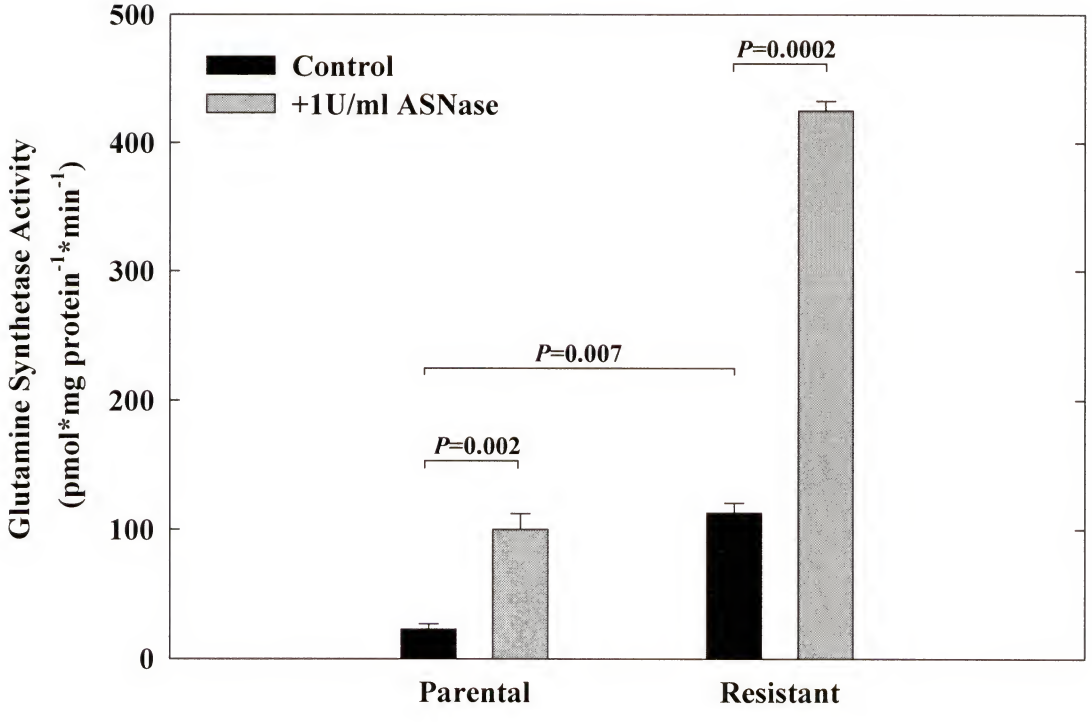

Parental
Resistant
Resistant
(6wk –ASNase)
Control
+ASNase
Control
+ASNase
Control
+ASNase
GS
18S
mRNA (Percent of Control)
160
140
120
100
80
60
40
20
0
*
1
2
3
4
5
6
Lane Number

Figure 4-10. Glutamine Synthetase Enzyme Activity in MOLT-4 Cells. The activity of GS in MOLT-4 lymphocytes was measured by culturing parental and resistant cells (1 x 104 cells/mL) for 12h in the absence (control) or in the presence (+ASNase) of 1U/mL ASNase, and performing an ion-exchange based enzyme assay as described in Chapter 2. The data are presented as pmol glutamine formed•mg^{-1}protein•min^{-1}, and a paired t-test was performed to verify significant differences between parental ± ASNase samples, parental and resistant control samples, and resistant ± ASNase samples. The data presented are the averages ± standard deviations (SD) of triplicate samples and are representative of two independent experiments.


Glutamine Synthetase Activity
(pmol*mg protein-1*min-1)
500
400
300
200
100
0
Control
+1U/ml ASNase
P=0.0002
P=0.007
P=0.002
Parental
Resistant

(Meister et al., 1974), and indicate that while GS mRNA content is only moderately altered by ASNase treatment, the enzymatic activity is highly responsive to asparagine or glutamine availability. Also, resistant cells appear to have a greater capacity for GS induction than parental cells. Whether this is a result of the constitutive up-regulation of GS mRNA, or the inability of parental cells to sufficiently induce GS within the 12 h treatment period is unknown, however, given the fact that resistant MOLT-4 cells have developed a phenotype of glutamine independence, it seems likely that continuous culture in the presence of ASNase has selected for enhanced GS expression.

Discussion

The experiments presented in Chapter 3 clearly indicate that the expression of AS is significantly enhanced in ASNase-treated resistant MOLT-4 lymphocytes as compared to parental cells. In addition, the basal level of AS expression is permanently up-regulated, resulting in constitutively high AS mRNA and protein levels in resistant cells cultured in the absence of ASNase for 6 weeks. Although there is a correlation between increased AS expression and ASNase resistance, it is possible that other metabolic factors are integral in rendering cells drug resistant. Given that the AS activity in ASNase resistant lymphocytes has been shown to be increased as much as 80-fold (Kiryama et al., 1989), and AS protein levels induced at least 50-fold in ASNase resistant MOLT-4 cells (Chapter 3), it was of interest to determine whether amino acid transport rates for aspartate and glutamine were enhanced in resistant cells to provide additional amino acid substrate for the AS reaction.

The data presented in this chapter represent the first documented observations of amino acid transport rates of parental and ASNase resistant human lymphocytes. Collectively, the data demonstrate that: anionic amino acid transport systems are not expressed in sensitive or resistant MOLT-4 lymphocytes, zwitterionic amino acid transport systems previously identified in human lymphocytes are present in MOLT-4 lymphocytes, the expression of these transporters is regulated by amino acid deprivation as a result of ASNase treatment, and the induced expression of System A transport is necessary for complete ASNase resistance. Furthermore, GS activity was found to be necessary for proliferation of MOLT-4 cells in the presence of ASNase, whereas transamination was necessary for cell growth in the amino acid fed state as well as during ASNase treatment.

MOLT-4 lymphocytes possess no detectable aspartate transport or anionic EAAT transporter mRNA expression, nor do they increase their intracellular aspartate concentration during ASNase treatment, even in the presence of approximately 400 μM extracellular aspartate, further confirming the absence of an active anionic transport system. These results are in contrast with several previous studies on ASNase resistance in non-ALL cells, indicating potential cell type-specific differences in the mechanism of ASNase resistance. For example, Broome (1968) documented that intracellular aspartic acid levels in parental 6C3HED mouse lymphoma cells rose 12-fold 3 h after ASNase treatment. Resistant tumor cells had a 3-fold increase in aspartate levels 3 h after treatment, and a 1.8-fold enhancement after 20 h. Other agents that produced cytotoxicity, including cyclosporamide or colchicine, did not significantly increase intracellular aspartic acid levels, indicating that this was an ASNase-dependent

phenomenon. Also, Keefer et al. (1985) observed that, upon treatment of mouse L5178Y lymphoma cells with ASNase, the intracellular aspartate content of parental and resistant cells was doubled within 60 min. Furthermore, Uggeri et al. (1995) determined that ras-expressing NIH3T3 mouse fibroblasts were more susceptible to ASNase treatment than non-transformed cells. The presumed mechanism for this increased sensitivity was a reduction in System $X^-_{A,G}$ transport and a corresponding decrease of intracellular glutamate in ras-expressing cells. Clearly, ASNase effects on metabolism must be interpreted in the context of the cell line used, since the presumed target of ASNase treatment, human leukemia cells, appear to express none of the anionic transport systems necessary for providing extracellular aspartate for the AS reaction.

In terms of the source of intracellular aspartate for the AS reaction, lymphocytes preferentially metabolize glutamate, generated by hydrolysis of glutamine, via transamination to produce alanine and aspartate, with the latter accounting for 70% of radiolabeled glutamine metabolized beyond glutamate (Newsholme et al. 1985). The aspartate produced by lymphocytes may primarily be utilized for the synthesis of the pyrimidine ring, however, it also may serve as a substrate for AS in MOLT-4 resistant cells. The data presented in Figures 4-6 and 4-7 using the transaminase inhibitor AOA confirm that general transamination is an important process in the growth of both sensitive and resistant cells. Interestingly, there appears to be a significant difference in the effect of AOA on sensitive and resistant cells. Whereas approximately 45% of sensitive cells undergo apoptosis and cell death when cultured for 48 h in the presence of AOA alone, resistant cells maintain almost complete viability, although cell growth is severely restricted (Figure 4-6,4-7). The mechanism for the survival of the resistant cells

exposed to AOA is unknown, although selection for growth in ASNase has resulted in the ability of these cells to avoid cell death in the absence of transaminase activity. Whether the resistant cells continue to be viable because of an alternate intracellular source of aspartate, or an enhanced anti-apoptotic phenotype in response to nutrient deprivation is unclear.

Although aspartate is a substrate of AS, it is primarily synthesized by the metabolism of another substrate, glutamine. The importance of glutamine to the survival of lymphocytes has been extensively reviewed by Newsholme et al. (1985), wherein several observations support the view that glutamine is essential for providing the lymphocyte with an ammonia source and carbon skeletons for biosynthesis, as well as energy during an immune stimulus. Lymphocytes express high levels of glutaminase, and this activity is increased by immunological challenge (Ardawi and Newsholme, 1982). Also, the utilization of glutamine in the rat mesenteric lymph node is increased after mitogenic stimulation with concanavalin A. The presence of extracellular glutamaine also is necessary for increased [^{3}H] thymidine incorporation after concanavalin A exposure, indicating that glutamine is essential for cell division of lymphocytes.

Based on the activities of enzymes involved in the metabolism of glutamine, Newsholme and colleagues (1982) have developed a model for glutamine utilization in lymphocytes, which is similar to that of the small intestine and kidney (Harrison and Parsons, 1977, Glodstein, 1976). The main products of glutamine metabolism in the lymphocyte are glutamate, aspartate, and ammonia, with minor amounts of asparagine, alanine, and lactate also produced. It is believed that glutamine is initially metabolized to

glutamate by glutaminase, rather than transamination, because the activity of glutaminase in lymphocytes is 57-fold that of glutamine transaminase (Newsholme, 1982). The reported low activity of glutamate dehydrogenase suggests that the glutamate formed is transaminated, rather than oxidatively deaminated via glutamate dehydrogenase (Newsholme, 1982). In an experiment not presented in the results, I observed that glutamate dehydrogenase mRNA was not detected in sensitive or resistant MOLT-4 cells, suggesting that the expression of this enzyme is quite low in these cells. The majority of the glutamate formed is transaminated to aspartate, based on the relative activities of aspartate and alanine aminotransferase. The activity of aspartate aminotransferase is 15-fold that of alanine aminotransferase in lymphocytes, accounting for the fact that this amino acid represents 70% of the glutamine metabolized past glutamate, as described previously. In terms of further oxidation of glutamine, the oxaloacetate formed by transamination of glutamate was converted to phosphoenolpyruvate by phosphhoenolpyruvate carboxy kinase (PEPCK), based on the inhibition of this enzyme by 3-mercaptopicolinate. Interestingly, it appears that the pyruvate formed is not converted to acetyl coenzyme A (acetyl-CoA) for complete oxidation via the Krebs cycle, rather it is metabolized to lactate, thus the oxidation of glutamine is thought to provide nitrogen and carbon for macromolecular synthesis under resting conditions (Newsholme, 1982).

In addition to macromolecular synthesis, glutamine derived from the extracellular environment is an important source of energy during immunological challenge, and, potentially, during ASNase treatment. Although glucose catabolism via anaerobic glycolysis is generally accepted as the major energy source for cultured cells, due to the

observed rapid rates of glucose conversion to lactate, several lines of evidence suggest that glutamine is a major oxidative fuel for a variety of cell types, including lymphocytes. Glutamine is not generally regarded as an essential amino acid in terms of human nutrition, however, Ehrensvärd et al. (1949) first demonstrated the essential nature of glutamine for individual cell types in tissue culture. As an outgrowth of this initial study, and those by Eagle (1959), who determined that glutamine requirements for cell cultures were in excess of 100-fold that of other amino acids, several investigators observed extensive utilization of glutamine in cell cultures, in some cases outstripping the oxidative rates of glucose by over 50-fold. The ability of glutamine to serve as a primary energy source for tumor cells was clearly demonstrated by Reitzer et al. (1979), who observed that HeLa cells proliferated at similar rates when glucose was replaced with galactose or fructose as the sugar source. Despite these similar growth rates, extreme differences in sugar utilization pathways were observed depending on the sugar source. During growth in glucose containing medium, most of the glucose was metabolized to lactic acid, with less than 5% of labeled glucose entering the citric acid cycle. However, analysis of metabolic intermediates indicated that during growth on fructose, no glycolytic intermediates were detected, with over 90% the fructose metabolized by the non-energy producing pentose phosphate shunt. An analysis of glutamine utilization reconciled these observations, indicating that in the absence of glucose, glutamine serves as the primary source of oxidative energy. These studies were extended to demonstrate that in the complete absence of sugar, HeLa cells, chick embro fibroblasts, rat hepatomas, and human diploid fibroblasts could proliferate normally if supplied with uridine or cytidine alone, indicating that the only necessary role for glucose in these cell lines is the

provision of ribose-5-phosphate for nucleic acid synthesis, with glutamine completely able to substitute as an energy source (Reitzer et al., 1979).

These observations, as well as studies of fibroblasts from a patient with succinyl-CoA;3-ketoacid-CoA transferase deficiency that exhibited an 80% decrease in glucose utilization with no reduction in growth rate, indicated that the utilization of glucose and glutamine undergo reciprocal regulation to maintain cell growth. Using human diploid fibroblasts, Zielke et al. (1978) documented a 5-fold increase in the CO_2 production from 1- and 5-^{14}C labeled glutamine during glucose starvation, whereas the addition of 5.5 mM glucose to the culture reduced glutamine oxidation by over 80%. Furthermore, repletion of log phase growing cells with 2 mM glutamine reduced glucose-6-^{14}C oxidation by 90%. Interestingly, glucose-1-^{14}C oxidation via the pentose phosphate shunt increased by 77% in the presence of glutamine, possibly indicating a regulatory role for this amino acid as an activator of purine synthesis.

Given that glutamine is essential for lymphocyte proliferation as well as serving as a substrate of the AS reaction, it was of interest to measure the transport rates of parental and ASNase resistant MOLT-4 cells for systems mediating the uptake of this amino acid. In accordance with other clinical and *in vitro* human lymphocyte studies, the majority of GLN uptake in parental MOLT-4 cells was mediated by System ASC. In addition, as presented in Figure 4-1, resistant cells cultured in the presence of ASNase had an approximately 4-fold increase in sodium-dependent glutamine uptake as compared to parental cells. Interestingly, System ASC, although not generally regarded as responsive to the extracellular concentration of substrate amino acids, exhibited significant increases in mRNA and transport activity as a result of ASNase treatment

(Figures 4-1, 4-2). Also, the activity of System A was enhanced approximately 3-fold in resistant cells maintained in ASNase-containing medium, consistent with the ability of lymphocytes to modulate the expression of this transport agency in the absence of substrate amino acids.

As expected with the enhanced expression of these transporters, the intracellular concentrations of the primary System A and ASC substrates alanine, glycine, serine, and threonine are increased in sensitive and resistant cells treated with ASNase. The intracellular concentration of another System A and ASC substrate, proline, is greater in parental cells treated with ASNase as compared to untreated controls, but lower in resistant cells. Interestingly, the basal level of proline in resistant cells is approximately 6-fold that of parental controls, but it is unclear what the role of this amino acid has, if any, in ASNase resistance.

To determine whether increased glutamine transport for asparagine production, macromolecular synthesis, or energy needs is a transient adaptive up-regulation, or a permanent genetic modification, resistant cells were incubated in the absence of ASNase for 12 h prior to transport analysis. As shown in Figure 4-1B and 4-2, the ASC activity and ASCT1 mRNA in resistant cells are reduced to the levels of parental control cells. Furthermore, the basal mRNA expression in resistant cells incubated in the absence of ASNase for 12 h are identical to that of resistant cells incubated in the absence of ASNase for 6 weeks, and parental control cells, indicating that no long-term up-regulation of ASCT1 gene expression occurs in these cells as a result of ASNase selection. Clearly, in contrast to the AS gene, the normal dynamic range of ASC adaptive

regulation is sufficient to provide the cell with additional amino acid transport without the need for permanent genetic overexpression.

Although System ASC expression returns completely to basal level upon the removal of ASNase after 12 h, System A appears to have a component of constitutive up-regulation in resistant cells. After removal of the drug for 12 h, System A transport continued to be active in resistant cells at a level almost 4-fold that of parental control cells, accounting for most of the additional glutamine transport activity observed in the resistant 12 h-ASNase cells. In contrast, the System A mRNA in resistant cells returns to a basal level of expression after 12 h of ASNase withdrawal, indicating that the up-regulated System A may be a post-translational effect, perhaps the result of decreased degradation of System A proteins or a reduction in transporter endocytosis.

The importance of up-regulated System A transport to ASNase resistance was demonstrated by inhibiting flux through this transport system by competitive inhibition with the non-metabolizable System A substrate, MeAIB. Upon treatment of parental cells with increasing concentrations of MeAIB, no decrease in cell growth or viability was observed, consistent with the low levels of System A activity in this cell line (Figure 4-5,4-1). In contrast, resistant cells exposed to saturating concentrations of MeAIB were rendered more sensitive to ASNase, as determined by a reduction in cell growth and viability in a concentration-dependent manner (Figure 4-5). Although there was only a modest level of apoptosis (7.4%) after 48 h of MeAIB treatment, later time points may have indicated a further increase in apoptosis. Clearly, these data indicate that increased System A activity is essential for cell growth of resistant cells in the presence of ASNase.

The effect of inhibition of System A by MeAIB has also been studied during liver regeneration after partial hepatectomy (PH) (Freeman et al., 1999). It was determined that the blockade of System A resulted in dramatic decreases in [^{3}H] thymidine incorporation and cell proliferation, indicating that System A was a rate-limiting factor in the growth of hepatocytes after PH. Interestingly, protein synthesis was not affected by System A inhibition, leading the investigators to conclude that the importance of a functional System A goes beyond simply providing substrate amino acids for general protein synthesis after PH. This may be a hepatocyte-specific observation, however, since alanine and glycine were determined to be unimportant factors in protein synthesis after PH (Brand et al., 1995).

Another potential role of System A is to regulate cell volume during proliferation. Changes in cell volume are associated with cell cycle, Na^+, K^+ and Cl^- transport, Na^+-dependent amino acid transport activity, and especially, System A (Bussolati et al., 1996). Amino acids function as osmolytes, increasing the cell volume when the intracellular concentration of amino acids rises. Although MeAIB is a compatible osmolyte for regulating cell volume, inhibition of System A with MeAIB results in a decrease in cell volume of hepatocytes after PH, whereas untreated controls increase in volume by 25% (Freeman, 1999). Taken together, these data indicate that, in hepatocytes, the inhibitory properties of MeAIB may be a result of undefined secondary factors that are affected by System A inhibition.

System L, due to its capacity for catalyzing the exodus of certain amino acids, has long been recognized as having a reciprocal regulation to that of systems A and ASC. In contrast to System A, uptake by System L is trans-stimulated by amino acids in the

intracellular space, resulting in a slight decrease in activity during general amino acid starvation (Moore et al., 1977; Shotwell et al., 1983). Consistent with these observations, ASNase treatment results in a transient down-regulation of System L in MOLT-4 lymphocytes, and a corresponding increase in the levels of intracellular leucine (Table 3-2). While glutamine transport via System L is quite low (Figure 4-1), the down-regulation of this carrier may serve to maintain the concentration of other metabolically important amino acids during ASNase treatment, including asparagine.

Although the transport systems for glutamine uptake are increased in activity during ASNase treatment, the intracellular concentration of glutamine in parental and resistant cells is markedly reduced during drug treatment. Most likely, this is a result of the depletion of extracellular glutamine by the glutaminase side reaction of ASNase, which occurs at a rate of approximately 2-3% of the asparagine hydrolysis reaction. Although a small percentage of the overall reaction, during clinical doses that typically generate ASNase levels in excess of 3U/mL serum, the glutaminase reaction is significant, resulting in the complete loss of plasma glutamine after 48 h (Miller, 1969). Although the plasma glutamine level of these patients recovered after a period of 5 days, presumably because of up-regulated GS activity, the glutaminase activity may be significant in terms of the anti-neoplastic activity of ASNase. Indeed, Eagle (1959) demonstrated the paradigm of glutamine dependence for HeLa cells. Cultured in glutamine and asparagine-free medium to simulate ASNase treatment, HeLa cells were unable to proliferate normally, however, the addition of supplemental asparagine to the medium had no effect on cell growth, indicating that glutamine was the essential component of the culture medium. This was confirmed by the observation that exposure

of HeLa cells to guinea pig serum, which has no glutaminase activity, had no effect on cell growth (Kim et al., 1968). Interestingly, culturing HeLa cells in increasing concentrations of ASNase for 6 months resulted in the outgrowth of cells resistant to the effects of ASNase. The addition of glutamic acid to the culture medium accelerated the rate of cell growth for resistant cells as compared to sensitive cells, implying that an increase in GS activity was responsible for the resistant phenotype, and that the supplemented glutamic acid served as a preferential substrate for this enzyme. This result also implies that HeLa cells, in contrast to MOLT-4 cells, have significant plasma membrane glutamate transport activity.

As discussed above, plasma levels of asparagine and glutamine of patients undergoing ASNase therapy are reduced to below detectable levels almost immediately after the initial administration of the drug, however, whereas asparagine remains completely repressed even after discontinuation of therapy, plasma glutamine levels begin to recover after approximately five days (Miller, 1969). These data indicate that GS activity is induced in tissues as a result of ASNase treatment *in vivo*. The relationship between GS activity and ASNase resistance *in vitro* was documented by Kiryama et al. (1989), who determined that U937 mouse lymphoma cells selected in ASNase had a 30% increase in GS activity as compared to parental cells. In addition, ASNase resistant U937 cells proliferated at a rate 50% faster than parental cells in glutamine-free medium. Interestingly, cells selected in asparagine deficient medium alone did not have an increase in GS activity, nor were they able to proliferate in glutamine-free medium, indicating that ASNase had selected for the ability to proliferate in a glutamine-deficient environment.

Upon analysis of GS enzyme expression in parental and resistant MOLT-4 cells (Figure 4-10), it was determined that parental and resistant cells undergo an approximately 4-fold increase in activity during ASNase treatment. The basal level of GS activity, however, is approximately 4-fold higher in resistant cells in the absence of ASNase, resulting in a greater absolute increase of GS activity than in parental cells. The induction of GS is primarily mediated by post-transcriptional mechanisms, because the GS mRNA levels of resistant cells were only 20% greater than parental cells. These results are consistent with the regulation of the liver-type GSII, being primarily accomplished by feedback inhibition of glutamine metabolites (Meister, 1974). Although the liver-type GSII is inhibited by serine and histidine, both of which are increased marginally by ASNase treatment, the release from inhibition is most likely accomplished by the severe reduction of the intracellular concentration of glutamine in both parental and resistant MOLT-4 cells. Furthermore, the reduction of GS in resistant cells after withdrawal of ASNase for 12 h may be the result of protein destabilization, catalyzed by an increased intracellular level of glutamine.

Although GS activity is reduced in resistant cells after 12 h of ASNase withdrawal, the activity is still heightened in relation to the basal expression seen in the parental cell population. In fact, the basal level of resistant cell GS activity is approximately equal to that of the ASNase-treated parental cells. While the 12 h time course may not be sufficient to completely repress GS activity in these cells, it is likely that a constitutive increase in GS activity occurs as a result of ASNase selection. Given that the GS mRNA levels are only slightly increased in resistant cells, this phenotype may arise from long-term changes in the post-translational expression of the enzyme, or

the less likely possibility that a mutation in the coding region that confers enhanced stability in the presence of its substrates.

The absence of System $X^-_{A,G}$ in MOLT-4 cells indicates that the glutamate for the GS reaction is likely not obtained from the extracellular medium, and must be synthesized intracellularly. Although proline, histidine, and arginine are capable of metabolism to glutamate via a series of transformations, the most direct route for synthesis is transfer of an α-amino group from a donor amino acid to α-ketoglutarate, forming glutamate. The potential need for transamination to provide GS with substrate during ASNase treatment further illustrates the necessity of these reactions in the viability of MOLT-4 cells.

The data regarding the cell growth in the absence of glutamine reveal an inherent similarity in terms of intracellular GS expression between parental and ASNase resistant cells. Both cell lines, when cultured in reduced extracellular concentrations of glutamine, become more dependent on intracellular glutamine synthesis via GS as monitored by sensitivity to the specific GS inhibitor, methionine sulfoximine (Figure 4-8). In the complete absence of extracellular glutamine, both cell lines are completely dependent on intracellular glutamine synthesis for viability, whereas when cultured in 2 mM glutamine, all of the glutamine requirements of the cell are provided by transport of medium glutamine and subsequent metabolism via glutaminase. These data for human MOLT-4 cells are in contrast, however, with those obtained in U937 mouse lymphoma cells by Kiriyama et al (1989), who showed that ASNase resistant cells proliferated at a faster rate than parental cells in the absence of extracellular glutamine.

Although the dependence on GS during glutamine deprivation is shared by parental and ASNase resistant cells, the cellular response to glutamine deprivation and GS inhibition is quite different between the two cell lines. Parental cells undergo significant apoptosis during exposure to MSO at low glutamine concentrations, while resistant cells appear to enter into a cytostatic state, neither proliferating nor undergoing any significant cell death. These data are similar to that of Papaconstantinou et al., (1998), who observed an induction of apoptosis in rat intestinal epithelium (RIE-1) cells deprived of glutamine. The withdrawal of methionine and cysteine, however, resulted in a reduction of cell growth without apoptosis. Also, Petronini et al., (1996) observed that a 12-24 h incubation in glutamine-free medium resulted in apoptosis of CEM, Namalwa, HL-60, and U937 lymphocytes. Interestingly, glutamine deprivation resulted in cell death independent of energy deprivation, as determined by intracellular ATP levels, whereas glucose starvation induced a drop in ATP and subsequent necrosis. Potentially, ASNase resistant cells possess anti-apoptotic mechanisms that may blunt the apoptotic cellular response to glutamine deprivation.

The availability of extracellular glutamine has been shown to alter the proliferation and cytokine production of lymphocytes. Also, lymphocyte function is impaired during glutamine deprivation, with macrophage phagocytosis being dependent on extracellular glutamine (Calder and Yaqoob, 1999). The imunosupressive effects of glutamine deprivation following sepsis, injury, burns, and surgery are likely due to the demand of glutamine from the liver, kidney, gut, and immune system, which outstrips glutamine release from the muscle (Calder and Yaqoob, 1999). Although the precise mechanism of glutamine growth modulation is not known, the addition of glutatathione

(GSH) to glutamine-starved cells resulted in an enhancement of cell proliferation, indicating that intracellular GSH levels may play a role in lymphocyte growth (Chang, 1999). Also, serum-starved HuH-7 hepatoma cells could be rescued from apoptosis by treatment with glutamine, which reduced intracellular levels of hydrogen peroxide, and enhanced intracellular GSH levels (Xy et al., 1997).

In summary, although AS expression is highly up-regulated in ASNase resistant cells, other metabolic factors changes also may contribute to ASNase resistance. First, zwitterionic amino acid transport is induced in resistant cells. This would be expected in light of the fact that ASNase depletes extracellular amino acids, however, it appears that enhanced System A transport is essential for complete ASNase resistance. Also, the down regulation of System L indicates that the cell attempts to retain accumulated amino acids, possibly including those serving as substrates of AS and asparagine itself. In addition to the enhanced transport of glutamine, resistant cells also exhibit an enhanced ability to survive in the absence of extracellular glutamine, most likely as a result of enhanced GS expression. Taken together, these data indicate that the cell modulates amino acid transport mechanisms and increases intracellular glutamine production to maintain ASNase resistance, and that these metabolic perturbations are essential for cell survival in the presence of the drug.

CHAPTER 5
SIGNALING PATHWAYS OF ASPARAGINE SYNTHETASE EXPRESSION

Introduction

Although the up-regulation of AS in response to amino acid starvation and ASNase treatment has been well documented, the precise mechanism by which mammalian cells respond to amino acids is largely unknown. Clearly, our understanding of nutrient control, and in particular the molecular signaling cascade of amino acid regulation, is vital to understanding the effects of ASNase on the cell, including the generation of ASNase resistance. Currently, the model of mammalian amino acid control can only be extrapolated from studies conducted in bacteria and yeast, however, some evidence is emerging indicating that specific signaling cascades in mammalian cells may be involved in amino acid control.

The initial signal for amino acid recognition in bacteria, yeast, and mammalian cells appears to be conserved across species, and consists of aminoacylated or deacylated tRNA synthetases. As reviewed in Chapter 3, control of specific bacterial operons is accomplished by a process called "attenuation", in which the availability of aminoacylated tRNAs dictates the expression of amino acid biosynthetic genes (Kolter and Yanofsky, 1982). In yeast, the expression of the yeast GCN4 transcription factor, a bZIP transcriptional activator which binds to the sequence 5'-ATGACTCAT-3' contained in the promoter region of amino acid regulated genes, also is dependent on a "sensing mechanism" for tRNAs. During periods of amino acid starvation, deacylated

tRNA binding to the carboxyl terminus of GCN2, a serine/threonine kinase which has homology to histidyl-tRNA synthetase, results in the phosphorylation of the initiation factor, eIF2 (Wek et al., 1989). The phosphorylation of eIF2 sequesters the necessary translation factor, eIF2B, which in turn results in the reduction of eIF2-GTP-$tRNA^{Met}$ complex formation, inhibiting subsequent binding to the 40S ribosomal subunit (Hershey, 1991; Merrick, 1992). The overall result of GCN2 activation by deacylated tRNA, therefore, is a reduction in overall translation initiation, but an increase in the translation of GCN4 due to a novel promoter sequence which actually enhances translation during periods of low initiation complex formation efficiency (Hinnebusch, 1997).

The intracellular level of tRNA synthetases also appear to initiate the signal for amino acid regulated genes in mammalian cells. Arfin et al., (1977) observed that AS expression was enhanced when a mutant CHO cell line containing a temperature sensitive mutant of asparaginyl tRNA was placed at nonpermissive temperature. This up-regulation could be reversed by repletion of the cells with the addition of exogenous asparagine. Also, cell lines containing temperature sensitive mutants of leucyl-methionyl- and lysyl- tRNA synthetases also demonstrated enhanced AS expression during incubation at nonpermissive temperature, further indicating that tRNA charging plays an essential role in the "sensing" of the amino acid signal.

Recently, a tRNA binding molecule analogous to GCN2 has been identified in mammalian cells (Wek et al, 1995) thus amino acid control in higher eukaryotes is potentially regulated at two different steps in translation initiation. The role of amino acids in translation initiation was first determined when rat livers perfused with amino

acid-deficient medium exhibited a density change of the 40S ribosomal subunit, as well as decreased methonine binding to the preinitiation complex (Flaim et al., 1982). As discussed above, the first regulated step of translation initiation in eukaryotic cells is the binding of methionyl-tRNA to the 40S ribosomal subunit, via the formation of the eIF2-GTP-$tRNA^{Met}$ ternary complex. The re-assembly of the eIF2-GTP-$tRNA^{Met}$ complex after ribosomal binding and concomitant GTP hydrolysis is regulated by the activity of eIF2B, a heteropentameric guanine nucleotide exchange factor which exchanges GDP for GTP from eIF2, promoting subsequent rounds of translation initiation. Kimball et al., (1988) observed that leucine and histidine starvation of L6 myeoblasts resulted in a significant decline of eIF2B exchange activity. In analogy to yeast, which sequesters eIF2B by phosphorylation of eIF2α, amino acid deprived L6 myeoblasts exhibited an increase in eIF2α phosphorylation. Refeeding of the cells with amino acids restored eIF2B activity and reversed the enhanced eIF2α phosphorylation (Kimball et al., 1988). In addition, perfusion of rat livers with histidinol in combination with histidine starvation resulted in a decrease in eIF2B activity, and an increase in eIF2α phosphorylation that was a result of a down-regulated eIF2α phosphatase (Kimball et al., 1991). Taken together, these data indicate that amino acids regulate global translation initiation by modulation of eIF2B activity via eIF2α phosphorylation.

The second regulated step in translation initiation is the binding of mRNA to the 43S preinitiation complex, mediated by the initiation factor eIF4E. eIF4E acts as a bridging protein, which interacts with the N^7-methylguanosine cap structure present on eukaryotic mRNAs, as well as the scaffolding protein eIF4G, which is bound to the 40S ribosome (Haghighat el a1., 1997). The affinity of eIF4E for the N^7-methylguanosine cap

is enhanced by phosphorylation (Minich et al., 1994) however, the availability of eIF4E also is controlled by association with eukaryotic initiation factor 4E binding protein 1 (4eBP1). In the repressed state, hypophosphorylated 4eBP1 sequesters eIF4E by strong binding, but phosphorylation of 4eBP1 on serine and threonine residues results in the release of eIF4E, allowing interaction with eIF4G and the formation of the 48S preinitiation complex (Lawrence and Abraham., 1997). The phosphorylation of 4eBP1 is mediated by FK506 binding protein-rapamycin-associated protein, or the mammalian target of rapamycin (FRAP/mTOR), which also is involved in the amino acid-dependent regulation of $p70^{S6k}$, a cell cycle-regulated kinase which phosphorylates the ribosomal protein S6 (S6) at five carboxy terminal serine residues (Bandi et al., 1993) (Figure 5-1).

The phosphorylation of S6 by $p70^{S6k}$ results in the enhanced translation of mRNA transcripts containing polypyrimidine tracts or extensive secondary structure in the 5' untranslated region (Jeffries et al., 1994; Terada et al., 1994). Members of this family of mRNAs include transcripts for ODC, ribosomal proteins, and translational elongation factors eIF1A and eIF2 (Jefferies et al., 1994). FRAP/mTOR directly phosphorylates $p70^{S6k}$ as well as 4E-BP1, and this activation is dependent on the availability of aminoacylated tRNA synthetases (Burnett et al., 1998; Iiboshi et al., 1999). Exposure of Jurkat cells to amino acid alcohols, as well as transfer of cell lines containing a temperature-sensitive mutant of histidyl-tRNA synthetase to nonpermissive temperature resulted in the deactivation and hypophosphorylation of $p70^{S6k}$ (Iiboshi et al., 1999). Also, treatment of cells with ASNase caused a reduction of $p70^{S6k}$ activity and 4eBP1 phosphorylation, indicating that withdrawal of amino acids has an inhibitory effect on the pathway (Iiboshi et al., 1999). Kimball et al., (1999) observed that leucine repletion

stimulated the phosphorylation of S6, and resulted in the enhanced translation of eIF4A. The specific inhibitor of FRAP/MTOR, rapamycin, completely blocked the leucine-induced phosphorylation of S6 and eIF4A synthesis, indicating that amino acid dependent signaling was mediated by the mTOR protein (Kimball et al., 1999). Interestingly, blockade of the FRAP/mTOR pathway with the PI3K inhibitor, wortmannin, had no effect on the amino acid dependent regulation of $p70^{S6k}$, indicating that the amino acid signal is transmitted to mTOR by a PI3K independent pathway (Iiboshi et al., 1999). Taken together, these data indicate that amino acids regulate the translation of specific mRNAs via the FRAP/mTOR signaling cascade, and although no tRNA binding site has been ascribed to the FRAP/mTOR protein, it is possible that aminoacylated tRNAs interact with a yet unidentified protein upstream of mTOR to affect translational control.

To date, no association between the mTOR signaling cascade and AS expression has been determined, however, an alternate signaling pathway involving protein kinase C (PKC), a serine/threonine protein kinase, may regulate AS transcription. Treatment of BALB3T3 cells with the phorbol ester TPA, an activator of PKC, resulted in a significant up-regulation of AS transcription (Chiyo et al., 1995). Although no further downstream elements were identified in this study, PKC activation may induce the phosphorylation and inactivation of inhibitor κB (IκB), resulting in the release of the transcription factor nuclear factor-κB (NF-κB) (Link et al., 1992). Also, PKC may activate Ras, a member of the mitogen activated protein kinase (MAPK) cascade by inhibition of GTPase activating protein (GAP), a negative regulator of Ras (Huang et al., 1993).

Figure 5-1. Mammalian Target of Rapamycin/$p70^{S6k}$ Signaling Cascade. $p70^{S6k}$ may be activated by the p110 catalytic subunit of PI3K, released from the p85 regulatory subunit by interaction with p80. Alternatively, $p70^{S6k}$ and 4E-BP1 may be directly activated by mTOR. Phosphorylation of $p70^{S6k}$ by a downstream kinase of mTOR results in the subsequent phosphorylation of the S6 ribosomal subunit, inducing protein translation and G1 progression. Phosphorylation of 4E-BP1 releases eIF-4E, an N^7-methylguanosine cap-binding subunit of the eIF-4F complex. eIF-4E is then free to associate with the eIF-4G/eIF-4F initiation complex, facilitating the translation of capped mRNAs. NF-κB also may be activated by PI3K via another downstream target of PI3K, AKT/PKB. The mTOR inhibitor, rapamycin, interacts with the cellular protein FKBP1, which in turn forms a ternary complex with mTOR. The PI3K branch of the cascade also may be inhibited by wortmannin. PI3K, phosphatidylinositol 3 kinase, AKT/PKB, protein kinase B, NF-κB, nuclear factor κB, TOR, mammalian target of rapamycin, $p70^{S6k}$, 70-kDa ribosomal S6 kinase, S6, ribosomal protein S6, eIF4E, eukaryotic initiation factor 4E, 4eBP1, eukaryotic initiation factor 4E binding protein 1.

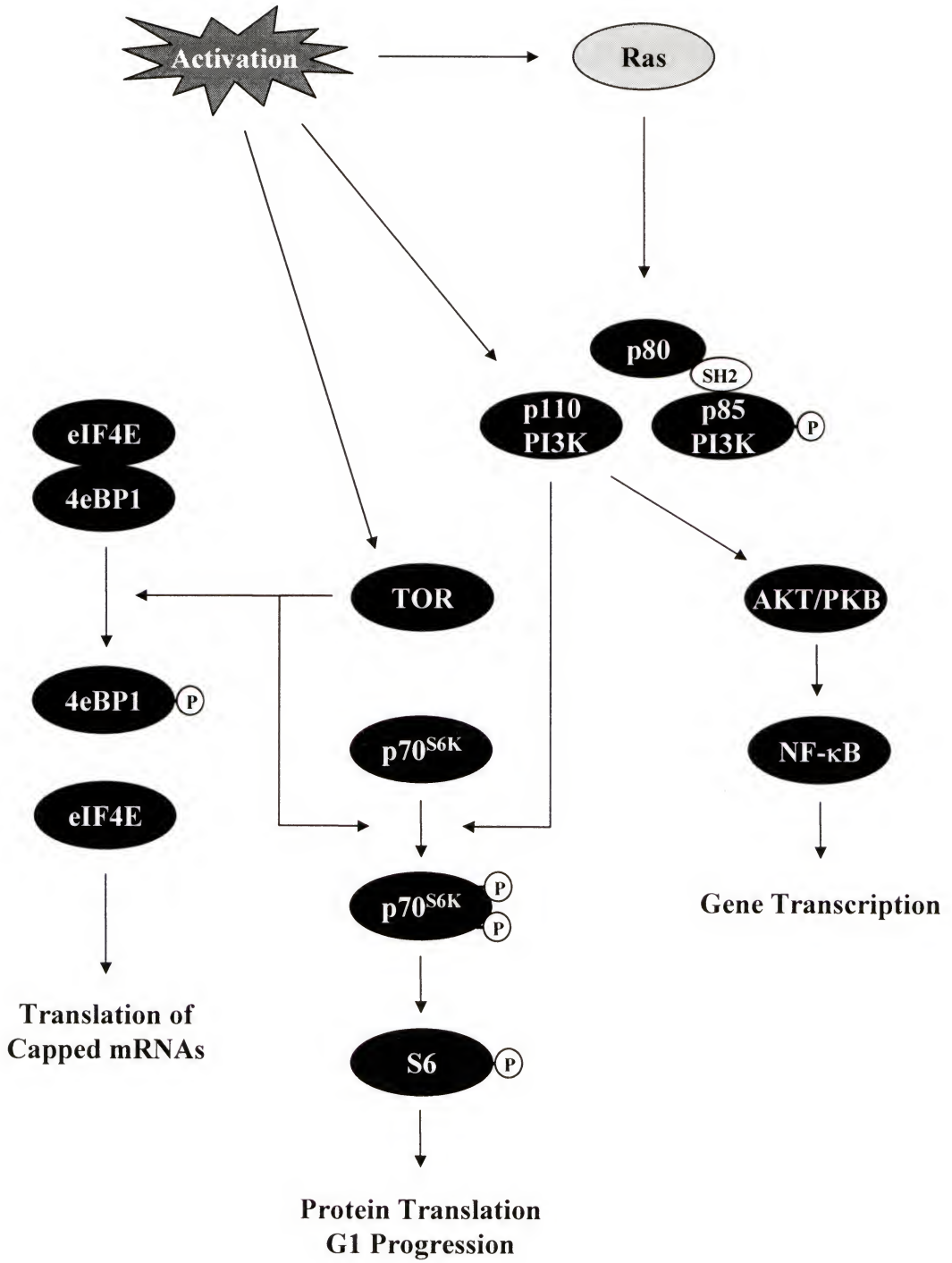
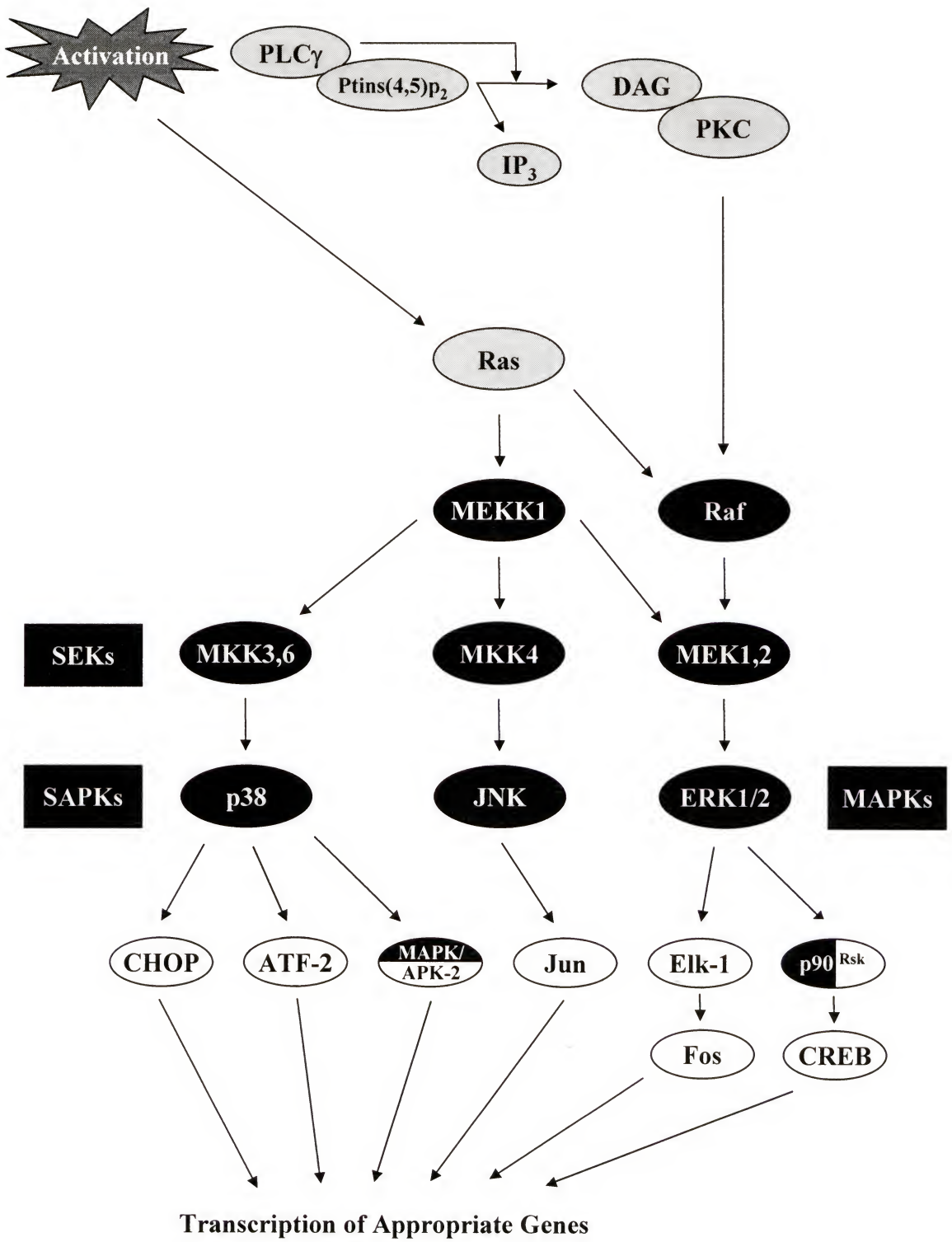

Activation
Ras
p80
SH2
p110
PI3K
p85
PI3K
P
eIF4E
4eBP1
TOR
AKT/PKB
4eBP1
P
p70S6K
NF-κB
eIF4E
p70S6K
P
P
Gene Transcription
Translation of
Capped mRNAs
S6
P
Protein Translation
G1 Progression

Figure 5-2. Mitogen Activated Protein Kinase Signaling Cascade (Adapted from Blalock et al., 1999). Activated PKC and the plasma membrane protein Ras activates the ERK1/2 pathway via Raf. ERK1/2 activates Elk-1 which, in turn, binds the SRE contained in the promoter of cytokine and immediate-early genes. ERK1/2 also interacts directly with $p90^{Rsk}$, which activates CREB, leading to the transcription of Act-1, a transcription factor which up-regulates cytokine and immediate-early genes. Ras also may initiate the activation of the SAPKs via MEK1. JNK phosphorylates the c-*jun* transcription factor. p38 activation leads to the phosphorylation of CHOP, ATF-2, and MAPKAPK2, which are active in the transcription of stress-responsive genes. The ERK1/2 pathway may be inhibited by PD98059, which interacts with MEK1,2. p38 may be specifically inhibited by SB203580. Currently, no pharmacological inhibitor for the JNK pathway is available. PLC-γ, phospholipase C-γ, $Ptins(4,5)p_2$, phosphatidylinositol diphosphate, IP_3, inositol triphosphate, DAG, diacylglycerol, PKC, protein kinase C. MEKK1, MEK1 kinase, MKK3,6, map kinase kinase 3,6, MKK4, map minase kinase 4, MEK1,2, mitogen activated extracellular regulated kinase 1,2, JNK, c-*jun* N-terminal kinase, ERK 1/2, extracellular regulated kinases 1/2, Elk-1, Ets related protein kinase 1, CHOP, CAAT/enhancer binding protein 1 homologous protein, ATF-2, activating transcription factor 2, MAPKAPK-2, mitogen activated protein kinase activating protein kinase 2, $p90^{Rsk}$, 90-kDa ribosomal S6 kinase, CREB, cyclic adenosine monophosphate response element binding protein, SEKs, stress/extracellular regulated protein kinases, SAPKs, stress activated protein kinases, MAPK, mitogen activated protein kinases.


Activation
PLCγ
Ptins(4,5)p2
DAG
PKC
IP3
Ras
MEKK1
Raf
SEKs
MKK3,6
MKK4
MEK1,2
SAPKs
p38
JNK
ERK1/2
MAPKs
CHOP
ATF-2
MAPK/
APK-2
Jun
Elk-1
p90 Rsk
Fos
CREB
Transcription of Appropriate Genes

The MAPKs, a group of three serine-threonine MAP kinases, including ERK1/2, $p38^{MAPK}$, and JNK, are activated by a variety of extracellular stimuli and stress responses (Figure 5-2). Typically, the activation of the MAPK cascade originates with a receptor tyrosine kinase (RTK), which, upon binding of its cognate growth factor, initiates a complex series of reactions which results in the communication of signals from the plasma membrane to the nucleus. The activated RTK binds the adaptor protein Grb2 by interaction with its Src homology 2 (SHC2) binding domain, which in turn binds the guanine nucleotide exchange factor (GNEF) Son-of-sevenless (Sos), via its Src homology 3 (SHC3) binding domain. Sos functions by exchanging GDP for GTP from Ras, a small, 21 kDa GTP-binding protein which remains tethered to the plasma membrane via a 15-chain fatty acid farnesylation that is attached to Ras by the enzyme farnesyl transferase (Waters et al., 1995; Pronk and Bos, 1994; Reiss et al., 1990). The GTP-bound form of Ras interacts with Raf-1, which in turn activates activated extracellular regulated kinase 1 (MEK1) by phosphorylation of two serine residues (Huang et al., 1993). After downstream activation of ERK1/2 by MEK1, ERK enters the nucleus and phosphorylates the transcription factor Ets related protein kinase 1 (Elk-1), which binds the serum response element (SRE), and also activates p90 ribosomal S6 kinase (Rsk), an S6 kinase that activates cAMP response element (CRE) binding protein (CREB) (Gille et al., 1995; Fisher et al., 1996; Xing et al., 1996). In terms of the potential involvement in nutrient control, CREB has been shown to be recruited to the promoter region of GRP78 following heat shock and okadaic acid treatment, as well as that of hydroxymethyl glutaryl CoA reductase, a key gene of intracellular cholesterol homeostasis (Bennett and Osborne, 2000).

Ras, via Rac, p21-activated kinase (PAK), and MEK1 kinase (MEKK1) also may stimulate the stress activated protein kinases (SAPKs), which are typically responsive to heat shock (Meier et al., 1996), UV exposure (Derijard et al., 1994), osmotic stress (Galcheva-Gargova et al., 1994), and inflammatory cytokines (Sluss et al., 1994), as well as ERK1/2, which is activated by growth factors and RTK phosphorylation. Activated MEKK results in the preferential activation of JNK>$p38^{MAPK}$>ERK1 and ERK2 (Zanke et al., 1996), via the serine/threonine and tyrosine stress/extracellular regulated kinases (SEKs) map kinase kinase 4 (MKK4), MKK3 and MKK6, and MEK1/2, respectively (Meier et al., 1996). The activation of JNK in turn activates the activating protein 1 (AP-1) transcription factor by phosphorylating serine residues 63 and 73 on the AP-1 component, c-*jun* (Hibi et al., 1993). $p38^{MAPK}$ is responsible for the activation of CCAAT/ehancer binding protein (C/EBP) homologous protein (CHOP) (Wang and Ron, 1996), activating transcription factor 2 (ATF-2) (Zhu and Lobie, 2000), and MAPK activating protein kinase 2 (MAPKAPK2), which is involved in the activation of heat-shock protein 27 (HSP27) (Rouse et al., 1994). Interestingly, HSP27 functions to destabilize mRNA cap binding complexes by interacting with eIF4G

Some studies have provided evidence that the MAPK signaling cascade also may be involved in nutrient control. Franchi-Gazzola et al., (1999) demonstrated that the amino acid-dependent adaptive induction of the System A amino acid transporter can be blocked by the MEK1 inhibitor, PD98059. However, the mechanism of ERK1/2 induction of System A is thought to be related to hypertonic stress, and not as a result of direct metabolic factors. The MAPK pathway also appears to be involved in the induction of GRP78. Treatment of 9L rat tumor cells with the $p38^{MAPK}$ inhibitor,

SB203580 completely inhibited the induction of GRP78 by heat shock and okadaic acid (OA) treatment (Chen et al., 1998). Based on EMSA supershift assays of the GRP78 promoter region, it was determined that ATF-2, induced by the $p38^{MAPK}$ pathway, heterodimerized with CREB, which was activated by the protein kinase A (PKA) pathway (Chen et al., 1998). Activation of GRP proteins as a result of glucose deprivation is mediated by the transcription factor ATF-6, indicating that functionally independent pathways may mediate GRP up-regulation as a result of heat shock or glucose deprivation. Currently, the involvement of the MAPK signaling cascade in the nutrient regulation of AS is unknown, however, the activation of CHOP by $p38^{MAPK}$ may provide a potential link to amino acid regulated genes.

CHOP is a bZIP nuclear protein which is related to the C/EBP family of transcription factors, and thus forms heterodimers with C/EBP proteins, acting as a dominant negative repressor of C/EBP binding (Ron and Habener, 1992; Fawcett et al., 1996; Friedman, 1996). C/EBP proteins function in the regulation of cellular proliferation (Cao et al., 1991), differentiation (Batchvarova et al., 1995), and programmed cell death (Zinszner et al., 1998), thus the expression of CHOP modulates a wide range of genes via its interaction with this protein. In addition, CHOP-C/EBP heterodimers may be recruited to a genomic element with a novel (3'-PuPuPuTGCAAT(A/C)CCC-5') core sequence, serving as a positively activating transcription factor (Ubeda et al., 1996). Recently, CHOP has been shown to interact with members of the AP-1 transcription factor family, *jun*D, c-*jun*, and c-*fos*, activating expression of the somatostatin, *jun*D, and collegenase genes (Ubeda et al., 1999). Phosphorylation of CHOP by $p38^{MAPK}$ significantly enhances the ability of the protein to

serve as a transcriptional enhancer (Wang and Ron, 1996). Taken together, the expression of CHOP leads to the inhibition of genes under the control of C/EBP, and the activation of a yet undefined set of genes, potentially involved in cellular proliferation, differentiation, and apoptosis.

Originally identified as a gene induced by UV exposure and alkylating agents (Fornace et al., 1989), CHOP has also been shown to be regulated by amino acids. Bruhat et al., (1997) observed that a variety of amino acids, including leucine, methionine, arginine, phenalanine, and threonine, when removed from an otherwise complete culture medium, resulted in the induction of CHOP mRNA in HeLa cells. The up-regulation of CHOP mRNA was ascribed to enhanced transcription as well as increased mRNA stability. Also, the induction was cycloheximide sensitive, indicating that the synthesis of an essential regulatory protein is necessary for CHOP mRNA expression (Bruhat et al., 1997). Although the factors responsible for CHOP induction during amino acid deprivation are not known, enhanced binding of the transcription factor ATF-4 was detected at the C/EBP-ATF binding site of the CHOP promoter region during arsenite treatment (Fawcett et al., 1999). Also, ATF-3 recruitment to the C/EBP-ATF site was enhanced during CHOP down-regulation, indicating that this protein may serve as a transcriptional repressor (Fawcett et al., 1999).

In addition to amino acid deprivation, CHOP also is particularly responsive to conditions which perturb the function of the endoplasmic reticulum (ER) (Zinszner et al., 1998). The ER, in addition to its widely-known function as a site of post-translational protein modification, folding, and oligomerization, has been identified as a major signal-transducing oraganelle which can direct the transcription and translation of genes that

relieve ER stress, including AS. The first observation of an ER-associated sensing mechanism was reported by Kozutsumi et al., (1988) who determined that the overexpression of a mutant influenza hemagglutin that was impaired in protein folding resulted in the expression of ER-resident proteins. Subsequently, a wide variety of conditions including glucose starvation, exposure to reducing agents, heavy metals, pharamacologic agents, and calcium ionophores have been shown to induce a set of protein chaperones and ER proteins including GRP78 and GRP94 (Resendez et al., 1985; Lin et al., 1986; Lee et al., 1984), protein disulfide isomerase (PDI) (Dorner et al., 1990), calreticulin (Llewellyn et al., 1996), and CHOP (Price and Calderwood, 1992). Given that the common stimulus for all of these treatments is thought to be the accumulation of misfolded proteins, this signaling pathway has been termed the unfolded protein response (UPR).

The UPR of *S. cerevisiae* has been extensively characterized, and is activated by conditions that overload the ER, including an accumulation of misfolded and aggregated proteins. The sensor for the yeast UPR is the ER resident protein inositol requiring and ER to nucleus signaling (IRE1/ERN1), a multi-domain, 1115-residue protein that consists of an amino-terminal 527 amino acid domain localized to the ER lumen, a 28 residue transmembrane sequence, and a carboxy-terminal domain which has homology to Ser/Thr protein kinases and RNaseL (Hanks and Hunter, 1995). Upon the detection of ER stress, presumably via the luminal domain, IRE1 is activated by oligomerization and trans-autophosphorylation (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). The activated IRE1 oligomer and the associated endoribonucleolytic activity of the IRE1

RNaseL domain subsequently function in the translational up-regulation of the UPR transcription factor homologous to ATF and CREB (HAC1).

HAC1p (Homologous to ATF and CREB) is a bZIP transcription factor that is required for the activation of UPR responsive genes, including GRPs and PDI-like genes, which catalyze protein folding (Nojima et al., 1994; Cox and Walter, 1996; Mori et al., 1998). During ER stress, HAC1p binds as a homodimer to a novel genetic element found upstream of all UPR-responsive yeast genes, which is termed the unfolded protein response element (UPRE) (Cox and Walter, 1996; Kohno et al., 1993; Nikawa et al., 1996). The UPRE sequence (5'-CANCNTG-3') is necessary and sufficient to mediate transcriptional induction in response to the UPR signal.

The activation of HAC1 is dependent on the translation of HAC1 mRNA to HAC1 protein. HAC1 mRNA is constitutively produced, and is detected on polysomes even in the absence of a UPR response, however, the 230 amino acid protein product of the full length mRNA is poorly translated (Chapman and Walter, 1997; Kawahara et al., 1997). During UPR stress, a 252-base intron is excised from the HAC1 mRNA by the IRE1 endoribonuclease activity, which relieves a translational block on the full length HAC1 mRNA (Chapman and Walter, 1997; Kawahara et al., 1997). The subsequent re-ligation of the HAC1 mRNA is not accomplished by the spliceosome, rather, tRNA ligase (Rlg1p) carries out the splicing reaction (Sidrauski et al., 1996; Sidrauski and Walter, 1997), which results in the excision of 10 amino acid residues and the addition of a novel 18 amino acid carboxy-terminus (Kawahara et al., 1997). The 238 amino acid HAC1 protein produced from the splicing reaction is efficiently translated from the mRNA, and functions as a transcription factor for UPR-responsive genes.

The mammalian UPR/ERSR, although not completely defined, appears to share many functional elements of the yeast UPR mechansm. Two IRE1 homologues, IRE1α and IRE1β, have been identified in human and mouse, however, the human homologue of HAC1 has not, as of yet, been isolated (Tirasophon et al., 1998; Wang et al., 1998). Missense mutations within the IRE1α kinase and endoribonuclease domains result in a 10-fold increase in IRE1α mRNA, indicating that the intracellular levels of IRE1α mRNA are controlled by the kinase and endoribonuclease activities of the IRE1α protein (Tirasophon et al., 1998). IRE1α is localized to the ER membrane, and preferentially associates with the nuclear envelope, co-localizing with the nuclear pore protein RanGAP1 (Tirasophon et al., 1998). In terms of functionality, IRE1α is capable of catalyzing the cleavage of yeast HAC1 at the 5' splice site at the identical site as yeast IRE1 (Tirasophon et al., 1998). Although IRE1α is unable to catalyze the cleavage of the 5' splice site, it has been postulated that IRE1α and IRE1β may interact as a heterodimer to cleave both the 3' and 5' splice sites of a putative homologue of the HAC1 mRNA.

In addition to the IRE1αβ/HAC1 pathway, the UPR in mammals can also be mediated by translational control through phosphorylation of eIF2α, in a manner analogous to the general control mechanism of amino acid control described for yeast (Hinnebusch, 1996). To promote translation of mRNA, an eIF2α-GTP-initiator $mRNA^{Met}$ ternary complex binds to the 40S ribosome, which then associates with the 60S ribosomal subunit, forming the 80S initiation complex. Polypeptide elongation is then commenced with the concomitant hydrolysis of GTP to GDP. To promote another round of initiation, the eIF2α associated GDP must be exchanged for GTP, however, phosphorylation of serine 51 of the eIF2 α subunit by a specific eIF2α kinase prevents

the release of the bound GDP, effectively halting further translation initiation (Pain, 1996). The eIF2α kinase that initiates the ER stress signal in mammalian cells has been identified as the ER resident protein, pancreatic eIF2α kinase (PERK). PERK is a multidomain protein with a luminal sequence similar to that of IRE1α, and β, fused to an eIF2α kinase motif that is capable of phosphorylating eIF2α at serine 51 (Harding et al., 1999). Agents that induce ER stress in mammalian cells, including treatment with tunicamycin, an inhibitor of *N*-linked glycosylation, or thapsigargin, an inhibitor of the ER-localized Ca^{2+}-dependent ATPase, result in the autophosphorylation of PERK, and the subsequent down-regulation of general translation initiation via eIF2α (Harding et al., 1999).

A third basic ER-nuclear signaling pathway that has been identified in mammalian cells involves NF-κB (Figure 1), a cytosolic transcription factor that is active in the inflammatory response. NF-κB exists as an inactive cytoplasmic complex bound to an inhibitory subunit, IκB (Baeuerle and Baltimore, 1988). During activation, the IκB kinases (IKK)α and IKKβ, along with NF-κB-inducing kinase (NIK), phosphorylate the IκB subunit on two serine residues, targeting the inhibitory subunit for degradation by the 26S proteasome (Beg et al., 1993; Traenckner et al., 1995). Upon degradation of the IκB subunit, five distinct NF-κB subunits (RelA, RelB, c-Rel, p50, and p52) hetero- and homo-dimerize, translocate to the nucleus, and induce transcription of target genes (Baeuerle and Henkel, 1994). Although generally characterized as a transcription factor which activates a number of genes involved in the immune and inflammatory response, NF-κB has also been implicated in a ER-nuclear signaling pathway. Meyer et al., (1992) observed that overexpression of the hepatitis B protein MHBS, which results in the

accumulation of proteins the ER, activates a NF-κB response. Subsequently, a number of pharamacological agents have been identified that induce the UPR and also result in NF-κB activation, including tunicamycin, 2-deoxyglucose, brefeldin A, and thapsigargin (Pahl and Baeuerle, 1995; Pahl et al., 1996). Interestingly, NF-κB activation may be distinguished from the UPR response by the glucosidase inhibitor castanospermine and 2-mercaptoethanol, which induce a UPR response, but have no effect on NF-κB activity. Also, treatment of cells with the phosphatase inhibitor okadaic acid or tumor necrosis factor (TNF)-α activate NF-κB, whereas GRP transcription is unaffected. Collectively, these data indicate that NF-κB mediates a signaling pathway distinct from that of the IRE1α and PERK UPR. Due to the fact that overexpression of wild type proteins induce NF-κB, but have no effect on the UPR, this pathway has been termed the endoplasmic reticulum overload response (EOR) (Liu et al., 1995).

The mechanism of NF-κB induction as a result of ER stress is mediated by the efflux of Ca^{2+} from the ER and a subsequent production of reactive oxygen intermediates (ROI). The generation of Ca^{2+} as a primary EOR signal has been determined from experiments indicating that pretreatment of cells with intracellular Ca^{2+} chelators 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) and BAPTA-AM completely inhibit the NF-κB EOR response (Pahl and Baeuerle, 1996; Pahl et al., 1996). The observation that the EOR response, regardless of stimulation, can be blocked by anti-oxidants, however, indicates that the release of Ca^{2+} precedes the production of ROIs, which, in turn activate NF-κB (Schrek et al., 1992; Pahl and Baeuerle, 1996).

The association between AS expression and ER stress has been demonstrated by Barbosa-Tessmann et al. (1999), who made the novel observation that AS was

transcriptionally induced by activation of the UPR with tunicamycin and azetidine-2-carboxylate (AZC), the latter, a proline analog that causes proteins to mis-fold. In addition, the response of the AS gene to ER stress was mediated by the identical *cis*-acting genomic elements required for induction by amino acid deprivation (Tessmann et al., 2000). Interestingly, the AARE originally identified by Guerrini et al., (1993) (5'-CATGATG-3'), through which the UPR signal was mediated, bears no similarity to the well-defined mammalian *cis*-acting element that mediates UPR-dependent transcriptional induction of GRP78, GRP94, calreticulin, and PDI isomerase (Yoshida et al., 1998; Roy and Lee, 1999). This UPR consensus sequence includes multiple copies of the endoplasmic reticulum stress element (ERSE) (5'-CCAT-N_9-CCAG-3'), located in a genomic region 5' from the coding sequence (Yoshida et al., 1998; Roy and Lee, 1999). Collectively, these data indicate that AS is a target of the UPR/ERSR signaling pathway, however, the genomic elements defined for AS induction via the UPR/ERSE may represent a novel motif that is responsive to both amino acid deprivation and ER stress. Currently, the relationship between the NF-κB/EOR response and AS is unknown, however, unpublished data from the Kilberg laboratory indicates that C/EBP, a downstream target of NF-κB, may be involved in the transcriptional regulation of AS.

The identification of the signal transduction cascades potentially involved in the up-regulation of AS as a result of ASNase treatment may have significant implications for the understanding of nutrient control and the strategy of pharmacological intervention for ALL. Currently, inhibitors of second messengers including Ras, Raf, and MEK are entering clinical trials for use as anti-proliferative agents (Adjei, 2000). Presumably, such compounds also may be useful in the reversion of ASNase resistance. The goal of

the experiments in this chapter, therefore, is to identify the potential signaling pathways responsible for the up-regulation of AS as a result of ASNase treatment and glucose deprivation. In particular, the involvement of the MAPK signaling cascade, which has not as of yet been studied in terms of nutrient control, and the mTOR/$p70^{S6k}$ pathway were investigated.

Results

To determine the signaling pathway(s) responsible for AS induction during ASNase treatment, MOLT-4 cells were pretreated for one hour with a signaling pathway inhibitor, then resuspended in fresh RPMI-1640 medium + 10% FBS containing the appropriate signaling inhibitor plus 1U/mL ASNase for 12 h. The cells also were resuspended in RPMI-1640 medium + 10% FBS medium lacking histidine and containing the appropriate signaling inhibitor to determine the effect of the withdrawal of an essential amino acid. To determine the signaling pathway(s) involved in AS induction as a result of glucose starvation, MOLT-4 cells were pretreated for one hour with a signaling pathway inhibitor, and then resuspended in fresh RPMI-1640 medium + 10% FBS lacking glucose and containing the appropriate signaling inhibitor for 12 h. Following all the incubations, total RNA from these samples was isolated and subjected to northern analysis as described in Chapter 2. The reduction in mRNA expression upon exposure to a particular inhibitor as compared to a mock treated control was taken as evidence that blockade of the specific pathway is involved in the up-regulation of the appropriate gene, although secondary effects from the inhibition of a signaling cascade with the potential for significant cross-talk also must be considered when interpreting the results.

To inhibit signaling via the ERK1/2 MAPK pathway, MOLT-4 cells were treated with PD98059, a specific inhibitor of MEK1, an upstream kinase of ERK1/2 (Figure 5-4, lanes 2, 8, 14 and 20) (Pang et al., 1995). In the control (fed) samples, there was a small, yet reproducible increase in AS expression when this compound was used, however, during ASNase treatment or histidine starvation there was approximately a 20% decrease in AS mRNA expression. No difference in AS mRNA between glucose deprived cells that were mock treated or exposed to PD98059 was observed. SB203580, an inhibitor of the $p38^{MAPK}$ pathway (Cuenda et al., 1995), had no effect on AS mRNA expression in the presence of ASNase, or in the absence of histidine (Figure 5-4, lanes 9 and 15). In the glucose-deprived cells, there was a 20% increase in AS expression as compared to mock treated glucose deprived cells (Figure 5-4, lanes 19 and 21).

In contrast to the modest effects seen during blockade of the MAPK signaling cascade, inhibition of the mTOR/$p70^{S6k}$ pathway with the mTOR binding compound, rapamycin, had a significant negative effect on induced AS mRNA expression. Treatment of MOLT-4 cells with rapamycin resulted in an approximate 60-70% decrease in AS expression when exposed to ASNase or deprived of histidine (Figure 5-4, lanes 10 and 16). Conversely, rapamycin treatment of glucose-deprived cells had minimal inhibitory effect when compared to the amino acid starvations (Figure 5-4, lanes 19 and 22), consistent with observations that indicate that the mTOR/$p70^{S6k}$ pathway is responsive to the intracellular concentration of amino acids (Iiboshi et al., 1999).

To examine the effect of PKC involvement in amino acid or glucose-dependent regulation of AS, the PKC inhibitor Gö6976 was used. Gö6976 is a specific inhibitor of the Ca^{2+}-dependent PKCα and $PKC_{\beta I}$ isozymes, with no effect on the Ca^{2+}-independent

δ-, ε- and ζ- isozymes (Qatsha, et al., 1993; Martiny-Baron et al., 1993). Treatment of MOLT-4 cells with this compound failed to inhibit AS mRNA accumulation during amino acid or glucose deprivation, indicating that PKCα or $PCK_{\beta I}$ is not involved in the starvation response in terms of AS expression (Figure 5-4, lanes 5, 11, 17 and 23). Interestingly, bryostatin-1, an activator of classical, Ca^{2+}-dependent PKC isoforms (Fields et al., 1989), potentiates the starvation response, enhancing AS mRNA expression during either amino acid and glucose starvation (Figure 5-4, lanes 6, 12, 18 and 24). Taken together, these data indicate that PKC may not initiate a starvation response, however, activation of PKC results in the further enhancement of AS transcription during amino acid or glucose deprivation.

To serve as a positive control that the glucose starvation response was functional in these experiments, the northern blot was stripped and re-probed with a probe for the glucose regulated protein, GRP78. GRP78 is a chaperone that facilitates the proper folding of proteins during periods of ER stress, and as such, is induced by treatments that perturb ER function, such as glucose deprivation (Kozutsumi et al., 1988). Comparing the fed control with the glucose-starved mock treatment (Figure 5-5, lanes 1 and 19), it is clear that glucose deprivation results in the induction of GRP78 mRNA, as expected. Interestingly, PD98059 and rapamycin seemed to have a modest inhibitory effect on GRP78 mRNA expression in both the basal and induced state. Bryostatin, as seen for the AS mRNA, appeared to have an potentiating effect on GRP78 mRNA when deprived of amino acids or glucose, with the greatest effect on the response to ASNase treatment. Surprisingly, the $p38^{MAPK}$ inhibitor SB203580 had no effect on GRP78 induction. Apparently, the signal for GRP78 induction as a result of nutrient deprivation is separate

from the signal generated by heat shock and okadaic acid (OA) treatment, for which Chen et al., (1998), observed ATF-2/CREB-mediated induction as a result of $p38^{MAPK}$ activation.

In addition to AS and GRP78, it was also of interest to examine the potential signaling pathway(s) involved in the up-regulation of another amino acid responsive gene, CHOP. CHOP is a transcription factor that is activated by UV exposure and alkylating agents (Fornace et al., 1989), mediated by the $p38^{MAPK}$ pathway (Wang and Ron, 1996). The purpose of this experiment, therefore, was to determine the signaling pathway of CHOP mRNA activation during nutrient deprivation. The data in Figure 5-5 indicate that the signaling pathway(s) mediating CHOP induction as a result of ASNase induction, histidine deprivation, and glucose starvation are similar to AS, although exposure to PD98059 had a more modest effect on CHOP expression than for AS, including the absence of CHOP mRNA in the PD98059 control fed sample (Figure 5-5, lane 2). As for rapamycin, this agent appears to have a negative effect on CHOP mRNA expression in response to amino acid deprivation, also as seen for AS (Figure 5-5, lanes 7, 10, 13 and 16). The data regarding the effect of rapamycin on CHOP expression due to glucose deprivation are unclear, due to the unusually large variations seen for these samples. Interestingly, the p38 inhibitor, SB203580, had no effect on CHOP induction during amino acid or glucose deprivation, indicating that the pathway involved in amino acid sensing is independent of that for UV exposure and alkylating agents. Because there is no avalable inhibitor for the JNK MAPK pathway, a mouse myeloma cell line (FDC-P1) that is stably transfected with a dominant-negative (*jun* N-terminal kinase) JNK signaling mutant was used to complete the study of the MAPK pathway

Figure 5-3. Signaling Pathways of AS mRNA Induction. To determine the signaling pathways of AS mRNA induction, parental MOLT-4 cells were pretreated for 1h with various signaling inhibitors, then incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase plus inhibitor for 12h before northern analysis was performed as described in Chapter 2. Additionally, the cells were transferred to histidine or glutamine deficient medium to identify AS signaling as a result of amino acid or glucose starvation. The cellular targets of each inhibitor and the concentration used were as follows: PD98059, ERK1/2, 50μM; SB203580, p38, 10mM; rapamycin, $p70^{S6k}$/mTOR, 10ng/mL; Gö6976, PKCα, 0.01μM; Bryostatin, PKCα activator, 0.01μM. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph.

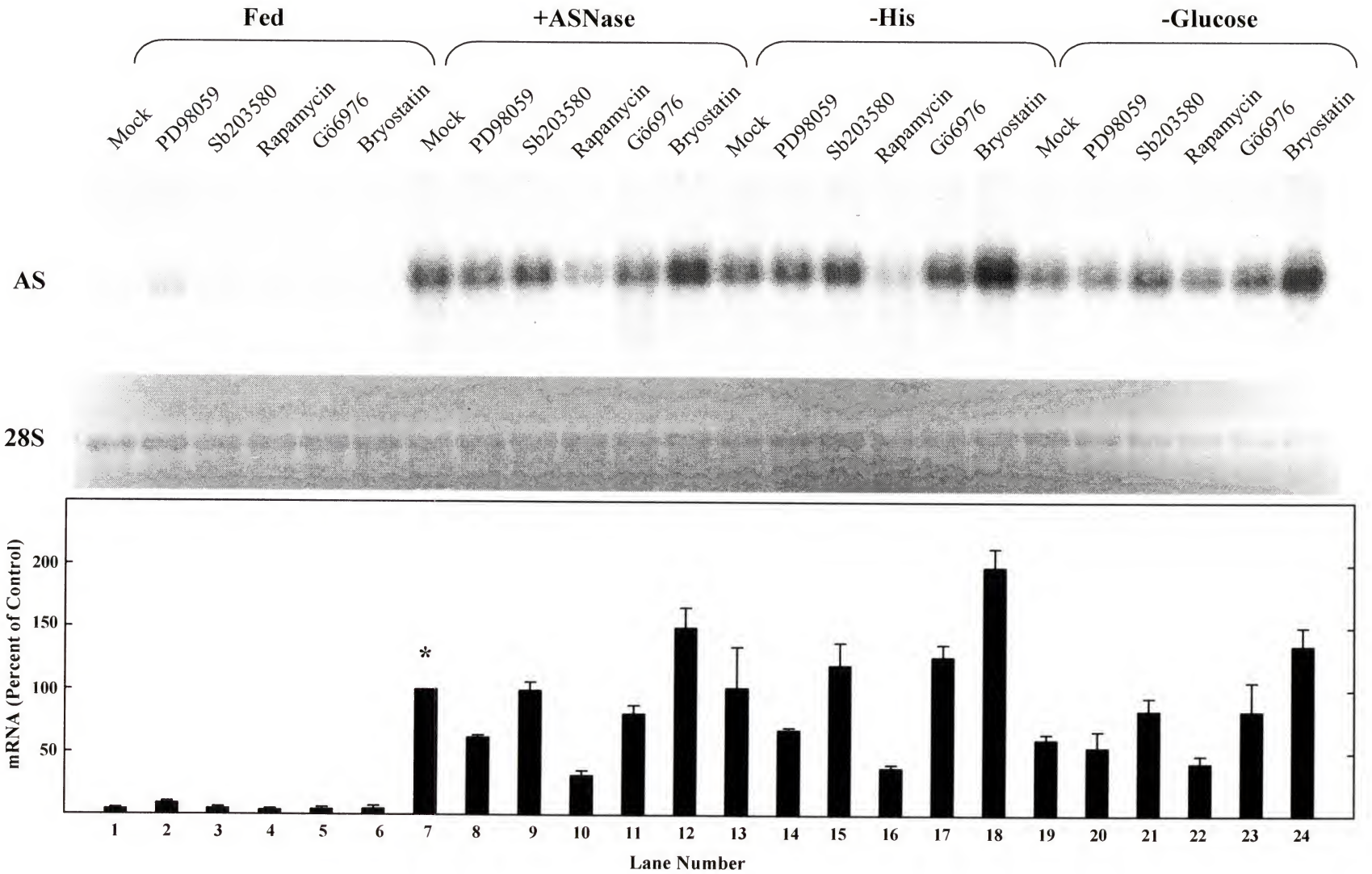

Fed
+ASNase
-His
-Glucose
Mock
PD98059
Sb203580
Rapamycin
Gö6976
Bryostatin
AS
28S
mRNA (Percent of Control)
200
150
100
50
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Lane Number

Figure 5-4. Signaling Pathways of GRP78 mRNA Induction. To determine the signaling pathways of GRP78 mRNA induction, parental MOLT-4 cells were pretreated for 1h with various signaling inhibitors, then incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase plus inhibitor for 12h before northern analysis was performed as described in Chapter 2. Additionally, the cells were transferred to histidine or glutamine deficient medium to identify GRP78 signaling as a result of amino acid or glucose starvation. The cellular targets of each inhibitor and the concentration used were as follows: PD98059, ERK1/2, 50μM; SB203580, p38, 10mM; rapamycin, $p70^{S6k}$/mTOR, 10ng/mL; Gö6976, PKCα, 0.01μM; Bryostatin, PKCα activator, 0.01μM. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human GRP78, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental cells incubated in the absence of glucose and plotted as a bar graph

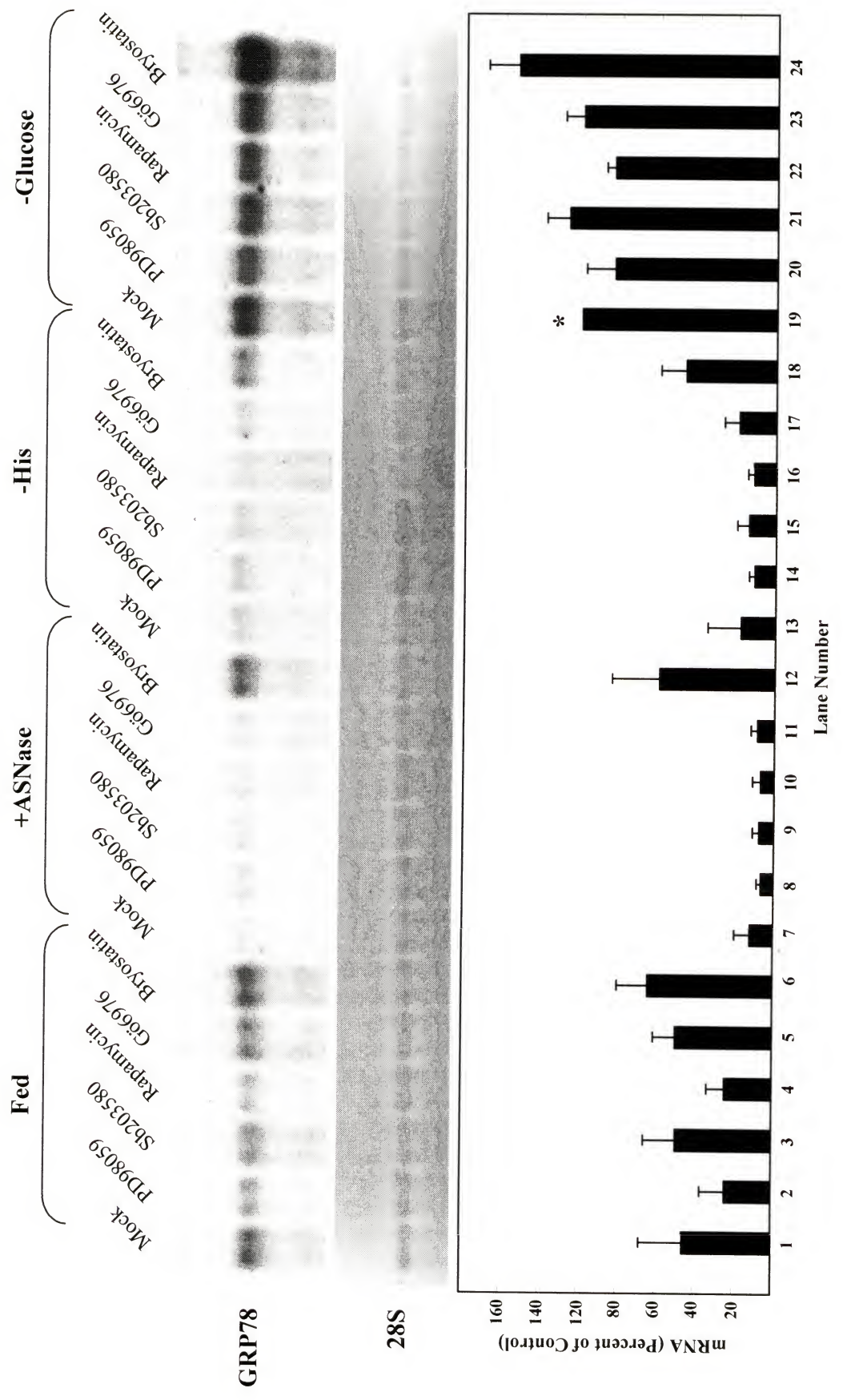

Fed
+ASNase
-His
-Glucose
Mock
PD980S9
Sb20358O
Rapamycin
Gö6976
Bryostatin
GRP78
28S
mRNA (Percent of Control)
Lane Number
*

Figure 5-5. Signaling Pathways of CHOP mRNA Induction. To determine the signaling pathways of CHOP mRNA induction, parental MOLT-4 cells were pretreated for 1h with various signaling inhibitors, then incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase plus inhibitor for 12h before northern analysis was performed as described in Chapter 2. Additionally, the cells were transferred to histidine or glutamine deficient medium to identify CHOP signaling as a result of amino acid or glucose starvation. The cellular targets of each inhibitor and the concentration used were as follows: PD98059, ERK1/2, 50μM; SB203580, p38, 10mM; rapamycin, $p70^{S6k}$/mTOR, 10ng/mL; Gö6976, PKCα, 0.01μM; Bryostatin, PKCα activator, 0.01μM. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human CHOP, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph.

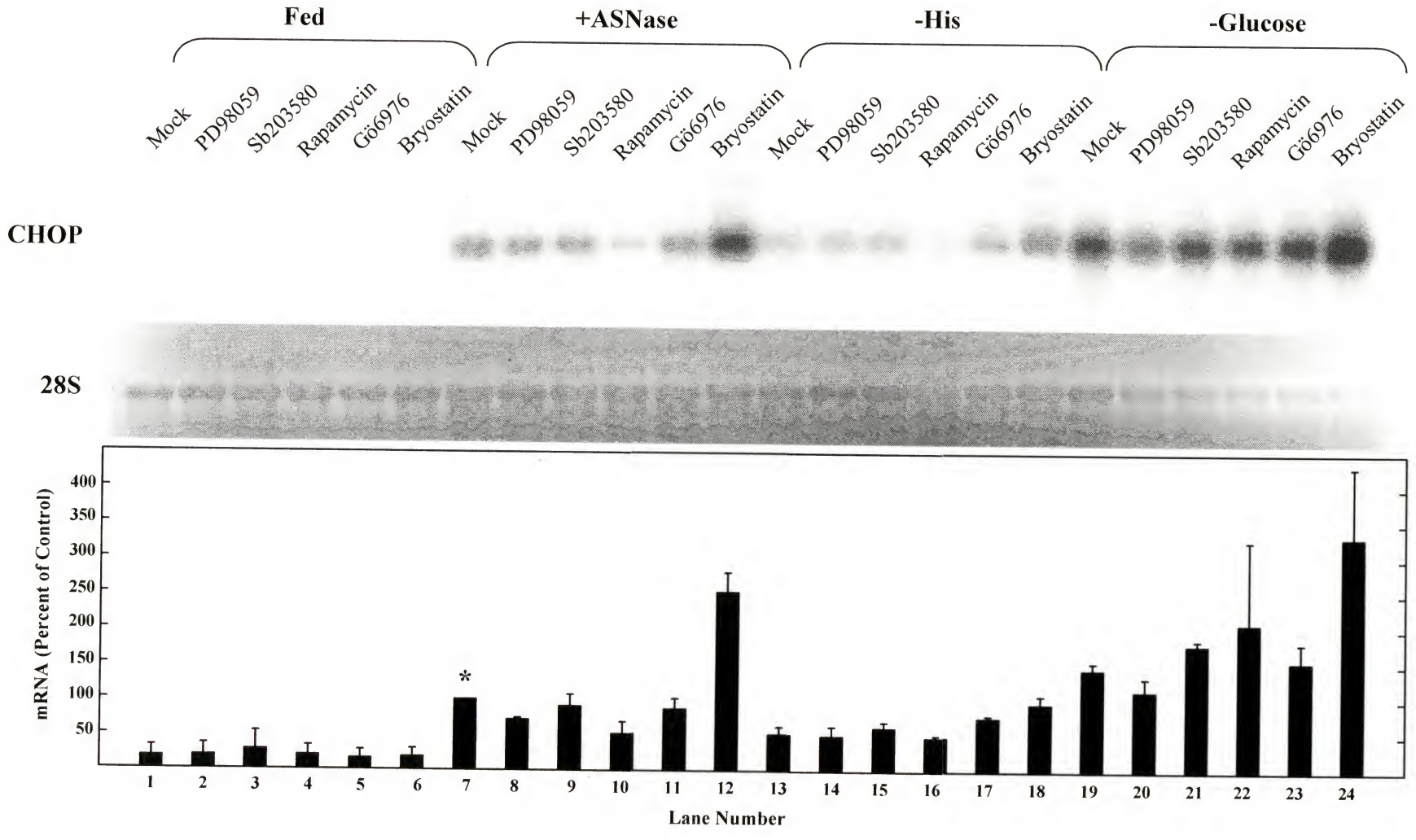

Fed
+ASNase
-His
-Glucose
Mock
PD98059
Sb203580
Rapamycin
Gö6976
Bryostatin
CHOP
28S
mRNA (Percent of Control)
400
350
300
250
200
150
100
50
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Lane Number

Figure 5-6. Asparagine Synthetase mRNA Induction in JNK Signaling-Deficient FDC-P1 Cells. To determine the involvement of the JNK pathway in AS mRNA induction, parental and JNK dominant negative (DN) FDC-P1 cells were incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase for 12h, upon which northern analysis was performed as described in Chapter 2. Additionally, the cells were transferred to histidine or glutamine deficient medium to identify JNK-mediated AS expression as a result of amino acid or glucose starvation. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human AS, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph.

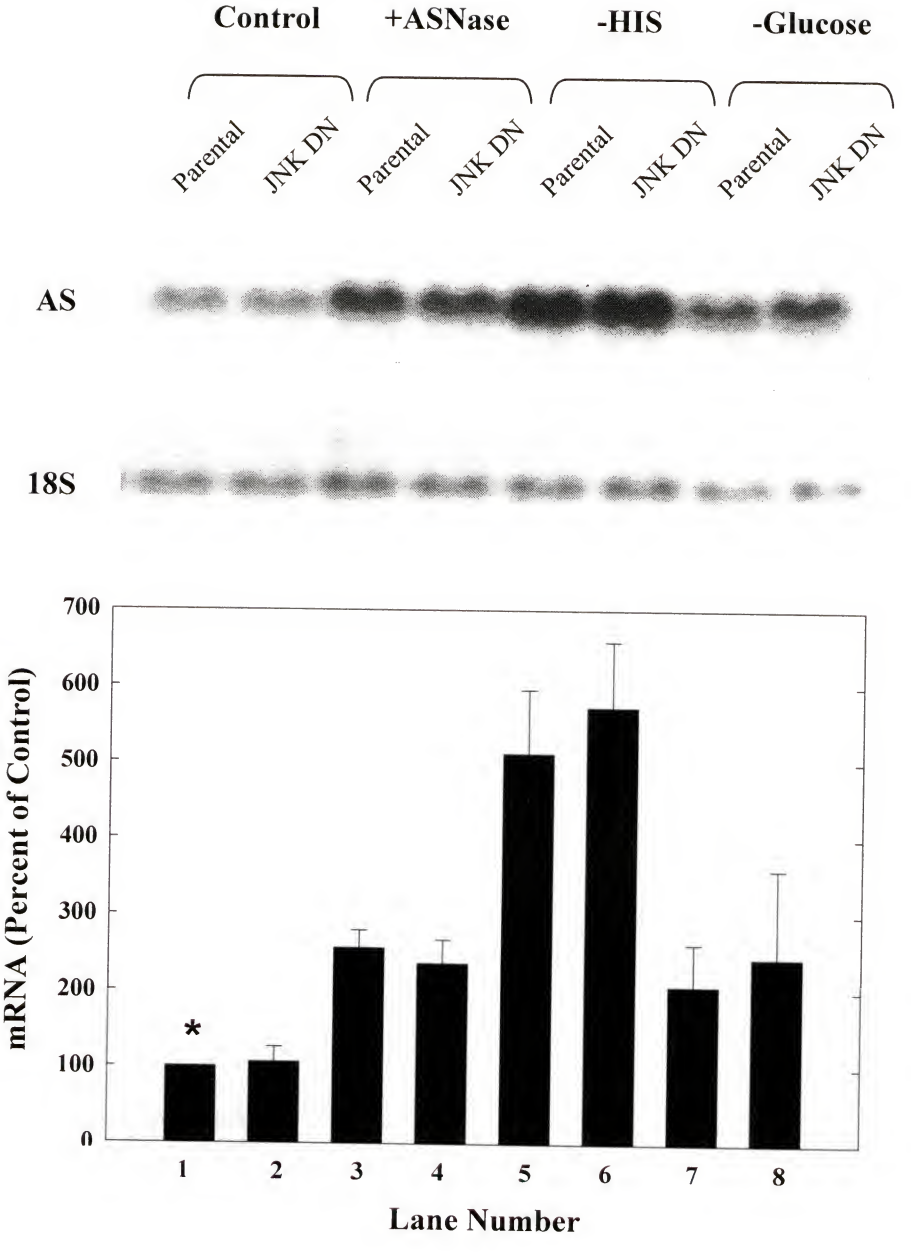
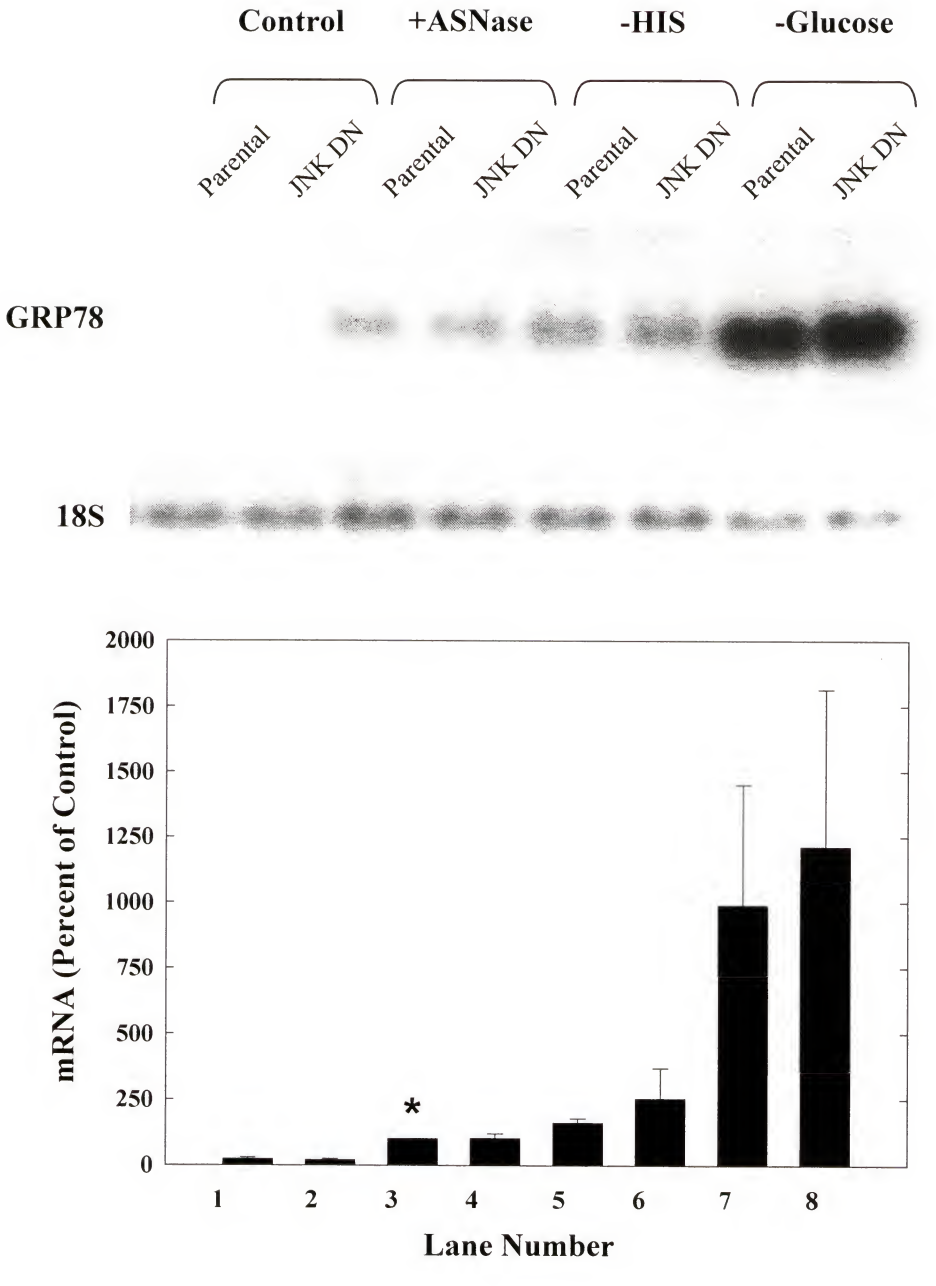

Control
+ASNase
-HIS
-Glucose
Parental
JNK DN
Parental
JNK DN
Parental
JNK DN
Parental
JNK DN
AS
18S
mRNA (Percent of Control)
700
600
500
400
300
200
100
0
*
1
2
3
4
5
6
7
8
Lane Number

Figure 5-7. Glucose Regulated Protein 78 mRNA Induction in JNK Signaling-Deficient FDC-P1 Cells. To determine the involvement of the JNK pathway in GRP78 mRNA induction, parental and JNK dominant negative (DN) FDC-P1 cells were incubated in the absence (control) or presence (+ASNase) of 1U/mL ASNase for 12h, upon which northern analysis was performed as described in Chapter 2. Additionally, the cells were transferred to histidine or glutamine deficient medium to identify JNK-mediated AS expression as a result of amino acid or glucose starvation. The blot (15μg/lane) was probed with a [^{32}P] radiolabeled cDNA corresponding to the coding region of human GRP78, with the ethidium bromide stain of the 18S ribosomal RNA serving as a measure of lane loading. The quantified data were normalized to parental cells incubated in the presence of ASNase and plotted as a bar graph.


Control
+ASNase
-HIS
-Glucose
Parental
JNK DN
Parental
JNK DN
Parental
JNK DN
Parental
JNK DN
GRP78
18S
mRNA (Percent of Control)
2000
1750
1500
1250
1000
750
500
250
0
*
1
2
3
4
5
6
7
8
Lane Number

during amino acid and glucose deprivation (Deng et al., 1998). As indicated in Figures 5-6 and 5-7, there is no difference in expression for AS, or GRP78 between the wild type and JNK-deficient cell line, indicating that this pathway is not involved in nutrient-dependent up-regulation of these genes. Clearly, if c-*jun* is necessary for amino acid or glucose induction of specific genes, it is activated by a separate pathway other than JNK.

Discussion

In this chapter, the impact of signaling pathway inhibitors on AS, CHOP, and GRP78 induction were examined. In general, blockade of the ERK1/2 pathway and mTOR $p70^{S6k}$ pathway had a negative effect on AS and CHOP transcription when cells were deprived of amino acids, but not glucose. This result is interesting, given the structural similarities between the amino acid responsive $p70^{S6k}$, and a downstream target of the ERK1/2 pathway, $p90^{Rsk}$. The p38 pathway did not affect AS transcription during amino acid or glucose withdrawal, which was unexpected, because the amino acid regulated gene CHOP appears to be activated by p38-mediated ATF-2 binding (Bruhat et al., 2000). The involvement of PKC in AS up-regulation was not detected, although activation of PKC appeared to potentiate the starvation signal. Finally, no reduction in AS expression was detected in amino acid deprived cells containing a dominant negative mutant of the JNK protein, indicating that this pathway is not involved in amino acid-dependent transcription of AS.

The targets of the ERK1/2 MAPK pathway include Elk-1 and $p90^{Rsk}$, with Elk-1 functioning in the transcriptional induction of c-*fos* by binding the SRE located in the c-*fos* promoter (Hipskind et al., 1991). In terms of amino acid regulation, glutamine (Rhoads et al., 1997) and glutamate (Vanhoutte et al., 1999) have both been found to

potently activate Elk-1 activation and c-*fos* transcription via the ERK1/2 pathway. Given these data, it is possible that blockade of the ERK1/2 pathway results in a reduction of the mitogenic signal from amino acids, and correspondingly up-regulates AS via unknown downstream factors. Indeed, in the fed state, AS transcription is enhanced during blockade of the ERK1/2 pathway.

Unfortunately, it is unclear why blockade of the ERK1/2 pathway would reduce AS transcription during amino acid, but not glucose withdrawal. In these experiments, the ERK1/2 pathway is inhibited by PD98059, however, the withdrawal of histidine or treatment with ASNase also may serve as a growth-inhibitory signal, so it is unknown why AS transcription would be negatively affected. It is possible, however, that two growth inhibitory signals may result in the onset of apoptosis rather than adaptive regulation, decreasing transcription of AS. Another possible explanation is a dual role of the ERK1/2 pathway in mitogenic activation as well as during a stress response.

$p90^{Rsk}$ is a serine/threonine kinase that is activated upon oncogenic transformation (Erikson et al., 1997), G1 cell cycle progression (Blenis, 1993), heat shock (Jurivich et al., 1991), and ionizing radiation (Kharbanda et al., 1994). The fact that cellular stresses as well as mitogenic signals activate $p90^{Rsk}$, indicate that transient mitogenic activation may be involved in the initial cellular response to stress, possibly including amino acid deprivation. Iiboshi et al. (1999) observed a transient increase in $p90^{Rsk}$ activity for approximately 1 hour upon withdrawal of amino acids, however, this increase was followed by a prolonged decrease in activity. Conversely, amino acid supplementation transiently decreased $p90^{Rsk}$ activity before returning to basal level after approximately 1 hr (Iiboshi et al., 1999). These data indicate amino acid withdrawal may elicit a transient

stress response in which $p90^{Rsk}$ may play a role. Blockade of the ERK1/2 pathway, therefore, may result in a lag of AS induction during ASNase treatment, since the initial stress response to amino acid withdrawal is blocked.

Interestingly, the N-terminal kinase domain of $p90^{Rsk}$, which is responsible for the activation of downstream substrates, has a 60% identity to the amino acid responsive $p70^{S6k}$ (Banerjee et al., 1990). The positions of the phosphorylation sites required for activation of $p90^{Rsk}$ are also similar to that of $p70^{S6k}$. MAPK catalyzes the phosphorylation of Ser^{364}, and Thr^{574} of the $p90^{Rsk}$ N- and C- terminal domains, respectively, upon which autophophosphorylation of the N- terminal Ser^{381} by the C-terminal domain results in activation of the N-terminal kinase and downstream phosphorylation (Dalby et al., 1998). In terms of downstream targets of $p90^{Rsk}$, activation of this kinase results in the serine phosphorylation of CREB. Phosphorylation of CREB at Ser^{133} occurs regardless of the type of stimulation, and is proposed to enhance DNA binding (Weih et al., 1990) and promote interaction with other members of the transcriptional activation complex (Chrivia et al., 1993). Phosphorylated CREB binds CREB-binding protein (CBP), a 265-kDa nuclear protein, and this interaction is necessary for the transcriptional activation of CREB, based on co-transfection experiments with CREB and CRE (Kwok et al., 1994).

Although the specific transcription factors necessary for the amino acid dependent up-regulation of AS have not been identified, the promoter region of the amino acid responsive CHOP gene has been shown to bind CREB family members during activation and down-regulation (Fawcett et al., 1999). Interestingly, CHOP has also been found to interact with AP-1 family members to activate specific AP-1 target genes (Ubeda et al.,

1999), however, the association between AP-1 and CHOP is unclear, because CHOP appears to have an opposing function as a dominant negative repressor of C/EBP transactivation, leading to programmed cell death. The role of the CHOP/AP-1 interaction, therefore, is proposed to be active directly following cellular stress during cell proliferation, when *fos* levels are high. In this model, CHOP/*fos* transactivation activates a subset of genes active in growth arrest and the apoptotic response (Ubeda et al., 1999).

Clearly, the influence of the ERK1/2 pathway on AS and CHOP expression will never be fully understood until the transcription factors involved in amino acid and glucose-dependent signaling are identified. Although the data appear contradictory between the ASNase treated and fed cells, for which an appropriate model cannot currently be envisioned, several interesting observations have been consistently identified. The most intriguing of these is the reduction of ASNase-induced AS mRNA expression in PD98059 treated cells, whereas this inhibitor has no effect on AS during glucose starvation. It appears that amino acids may somehow influence AS expression as a result of amino acid withdrawal, however, the signal for glucose starvation interacts with the AS transcriptional factors in a manner independent of the ERK1/2 pathway.

Although no evidence of AS expression during JNK blockade was observed in the FDC-P1 cell line, Rhoads et al., (2000) showed that the JNK pathway was activated when glutamine starved rat IE-6 intestinal cells were re-fed glutamine, indicating a role for this pathway in the amino acid response. The lack of differences in AS expression between FDC-P1 wild type and dominant negative cell lines indicates that AS is not up-regulated during the fed state as a result of JNK blockade, nor is the JNK pathway involved in an

amino acid starvation-induced stress response that affects AS transcription. Either there are cell-type differences in the role of JNK signaling, or the JNK pathway mediates the amino acid-induced signal to different genes other than AS.

Blockade of the SAPK p38 pathway had an unexpected lack of inhibition of AS and CHOP transcription upon ASNase treatment or histidine withdrawal, based on the observation that the p38 pathway has been shown to mediate the phosphorylation of CHOP, and that this phosphorylation is necessary for the activation of this protein (Wang and Ron, 1996). Furthermore, ATF-2, a downstream target of p38 has been shown to bind the promoter region of CHOP, further indicating the central role of this pathway in CHOP activation (Bruhat, unpublished data). Why the blockade of this pathway did not inhibit the AS or CHOP transcriptional response to amino acid or glucose deprivation is unclear. One possible explanation is that this pathway is not involved in nutrient sensing, and that the p38 pathway mediates CHOP activation in response to stimuli other than amino acid or glucose starvation.

The involvement of the mTOR/$p70^{S6k}$ pathway in amino acid dependent transcription of AS and CHOP was examined using the specific mTOR inhibitor, rapamycin. The involvement of the mTOR pathway in AS transcription was of interest because $p70^{S6k}$ is responsive to the intracellular concentration of amino acids (Kimball et al., 1999; Hara et al., 1998). Refeeding of leucine from leucine-deprived L6 myoblasts resulted in the phosphorylation and activation of $p70^{S6k}$ and ribosomal protein S6, as well as phosphorylation of eIF2α. mTOR was identified as mediating the amino acid-dependent response, because rapamycin completely blocked the effect of leucine refeeding (Kimball et al., 1999). Also, withdrawal of amino acids from CHO cells

induced the deactivation of $p70^{S6k}$ and dephosphorylation of eIF4E-BP1, whereas refeeding with amino acids increases $p70^{S6k}$ activity, further illustrating the responsiveness of this pathway to the intracellular concentration of amino acids (Hara et al., 1998). In agreement with this finding, ASNase treatment results in the inhibition of phosphorylation of the $p70^{S6k}$ substrates, ribosomal proteins S6 and S17 (Patel et al., 1996).

In terms of AS, inhibition of the mTOR pathway appears to reduce the transcriptional response of AS when MOLT-4 cells are deprived of amino acids, but not glucose. In experiments performed by Kimball et al. (1999) and Hara et al. (1998), cells were deprived of amino acids, which reduced the phosphorylation of $p70^{S6k}$, and then treated with rapamycin before refeeding, inhibiting the re-activation of the pathway. The experiments performed in this chapter were slightly different, in that rapamycin was applied to the cells in a fed state, then starved of histidine, glucose or exposed to ASNase. One might predict that upon amino acid withdrawal, the down-regulation of mTOR/$p70^{S6k}$ would signal the cell to halt translation of ribosomal proteins, and would, by analogy to the GCN4 protein which is translationally up-regulated during amino acid starvation, up-regulate yet unidentified transcription factor(s) to mediate the amino acid response and consequently, up-regulate AS transcription. However, the converse was observed. In the fed state, no accumulation of AS mRNA was detected in MOLT-4 cells treated with rapamycin, which may indicate that dephosphorylation of $p70^{S6k}$ does not lead to the induction of AS mRNA. Potentially, the factors involved in AS up-regulation are not affected by $p70^{S6k}$ activity. On the other hand, there was a reproducible decrease in CHOP and AS expression during amino acid, but not glucose withdrawal, indicating

an amino acid-specific response. As with the ERK1/2 pathway, perhaps a certain amount of $p70^{S6k}$ deactivation leads to an adaptive response, but amino acid withdrawal as well as rapamycin treatment results in the onset of programmed cell death, with a concomitant loss of nutrient responsiveness. The specificity of the mTOR/$p70^{S6k}$ pathway is clear in this regard, because glucose starvation in combination with rapamycin treatment does not trigger a putative apoptotic response. Further studies linking the mTOR pathway to AS expression will help elucidate the mechanism of the response in MOLT-4 cells.

The examination of the PKC pathway as a potential mediator of AS up-regulation was initiated by the study of Chiyo et al., (1995), who determined that AS mRNA levels were significantly enhanced upon treatment of BALB 3T3 cells with TPA, a direct activator of PKC. Treatment of MOLT-4 cells with Gö6976, a specific inhibitor of the Ca^{2+}-dependent PKCα and PKC$_{\beta I}$ isozymes, (Qatsha, et al., 1993; Martiny-Baron et al., 1993), resulted in no inhibition of AS transcription as a result of amino acid withdrawal or glucose starvation. However, when cells were treated with bryostatin-1, an activator of classical, Ca^{2+}-dependent PKC isoforms (Fields et al., 1989), a potentiation of the stress response was generated. In contrast to the data obtained by Chiyo et al. (1995), no activation was observed in bryostatin-treated fed cells, so the PKC response in these cells appears to be restricted to amplification of the starvation response. A potential mechanism for this response may be related to the release of intracellular Ca^{2+}, mediated by IP_3, the product of DAG.

The transcription factor NF-κB is activated by the UPR in response to Ca^{2+} release from the ER. Potentially, Ca^{2+} release as a result of PKC activation could similarly induce NF-κB, and up-regulate AS in a manner analogous to the UPR. The fact

that bryostatin does not result in the up-regulation of AS or CHOP during the fed state may indicate that a "permissive" factor that is present only during amino acid or glucose starvation allows transcription of these genes. Interestingly, Tessmann et al., (2000) recently identified genomic elements essential for AS induction that were utilized similarly for glucose and amino acid starvation. The observation that CHOP and AS are potentiated by bryostatin during both amino acid and glucose starvation may indicate that the putative "permissive" elements that allow a nutrient response are similar in both instances.

CHAPTER 6
CONCLUSIONS AND FURTHER DIRECTIONS

Conclusions

The research presented in this thesis describes several nutrient adaptive mechanisms in which MOLT-4 leukemia cells respond to ASNase treatment. The most striking aspect of ASNase treatment is a transcriptional induction of the AS gene. In parental MOLT-4 cells sensitive to ASNase, there is a significant induction of AS during drug exposure, indicating that ALL leukemia cells are capable of responding to nutrient deprivation. For MOLT-4 cells resistant to ASNase, the AS mRNA levels remain elevated, even after 6 weeks of continuous culture in the absence of the drug. These data indicate that the AS gene is permanently up-regulated in resistant cells, and that the high basal level of AS may be essential for the maintenance of ASNase resistance. In agreement with this hypothesis, the expression of AS in parental MOLT-4 cells significantly decreased ASNase-induced cytoxicity.

Because the AS gene has been documented to be transcriptionally up-regulated in response to the depletion of several different amino acids, with the greatest induction elicited by the absence of histidine (Hutson et al., 1997), experiments were performed to determine whether the expression of AS was maximally expressed during ASNase treatment. In parental MOLT-4 cells, the induction of AS mRNA during ASNase treatment was similar to that observed during histidine withdrawal, indicating that in this cell line, AS was maximally induced by ASNase. In contrast, histidine withdrawal

produced a greater AS mRNA induction in resistant cells than ASNase treatment, indicating that the up-regulation of AS in resistant cells in response to ASNase does not represent the full capacity of the cell to respond to amino acid depletion.

In addition to the regulation of AS, several metabolic changes also were observed between parental and resistant MOLT-4 cells that may contribute to ASNase resistance. The neutral amino acid transporters Systems A and ASC were enhanced in ASNase resistant cells, and the up-regulated System A activity was shown to be essential for complete ASNase resistance. In addition, System L, a transport agency which could potentially function in the efflux of amino acids was down-regulated in ASNase resistant cells. Taken together, these data indicate that the cell coordinately regulates plasma membrane amino acid transport to provide additional amino acid substrates, possibly for the enhanced AS found in resistant cells. An important distinction between the transport regulation and that of AS to be noted is that of reversibility. The expression of AS remained at a high level in ASNase resistant cells, even in the absence of ASNase for 6 weeks, implying a permanent genetic modification. The transport data, however indicate that a transient up-regulation of transporter activity occurs in resistant cells that is largely reversible upon ASNase withdrawal, implying that the normal dynamic range of transport regulation is sufficient to provide the cell with sufficient amino acid substrate during ASNase treatment.

In addition to changes in plasma amino acid transport, differences in the metabolism of the AS substrates, aspartate and glutamine, were identified between parental and resistant cells. Although transamination was necessary for the growth of both parental and ASNase resistant cells, the general transaminase inhibitor AOA had a

cytostatic effect on resistant cells, as opposed to a cytotoxic action on parental cells. In terms of glutamine metabolism, the intracellular synthesis of glutamine becomes necessary for cell viability during glutamine limitation, as would be the case during ASNase treatment. Consistent with this observation, the up-regulation of GS has been documented to be increased in parental and resistant cells following ASNase exposure, however, the ASNase resistant cells had a four-fold increase in basal and induced GS activity than parental cells. This increased GS activity may indicate why, during treatment with the specific GS inhibitor MSO, parental cells underwent apoptosis and cell death, whereas ASNase resistant cells were cytostatic, with comparatively little cell death. Taken together, these data indicate that ASNase resistant cells have changes in plasma membrane transport and metabolism that may contribute to ASNase resistance. Considering the observation that AS expression alone is sufficient for ASNase resistance, it is possible that these perturbations are transient, and that the only permanent modification necessary for full ASNase resistance is AS. However, it does appear that there are basal differences in GS expression, and the ability of cells to avoid apoptosis during nutrient stress between parental and ASNase resistant cells. Asparaginase resistant cells may primarily depend on increased AS for ASNase resistance, but metabolic adjustments and yet undefined anti-apoptotic mechanisms also may contribute to the drug resistant phenotype

Because AS expression is critical to ASNase resistance, it was of interest to determine the signaling pathways that contribute to AS up-regulation during nutrient deprivation. When MOLT-4 cells were treated with the mTOR/$p70^{S6k}$ pathway inhibitor, rapamycin, amino acid, but not glucose-induced AS expression was inhibited. Also,

amino acid-induced AS expression was reduced by the ERK1/2 inhibitor, PD98059, although to a lesser degree than rapamycin. These data indicate that the mTOR and ERK1/2 signaling pathways may be important in nutrient sensing during ASNase treatment, and that the pathways are specific for amino acid, but not glucose responsiveness.

In conclusion, ASNase resistance is acquired by several nutrient adaptive mechanisms. Interestingly, all of the genes studied that contribute to ASNase resistance appear to be regulated in a distinct manner. The expression of AS occurs transcriptionally, and is maintained at a high level, even after the withdrawal of ASNase for 6 months. Plasma membrane transport also is regulated transcriptionally, but is transiently induced, generally returning to basal mRNA activity levels after 12h of culture in ASNase-free medium. GS is regulated post-transcriptionally, however, there appears to be a component of long-term up-regulation in ASNase resistant cells. Taken together, the data indicate that, although AS expression is primarily responsible for ASNase resistance, MOLT-4 leukemia cells employ a variety of regulatory mechanisms to maintain drug resistance.

Future Directions

In terms of further directions, this project may be expanded in a number of ways to increase our current understanding of ASNase resistance, and perhaps, drug resistance in general. As far as the current *in vitro* cell line model is concerned, it would be informative to obtain more precise data on the mechanism of AS up-regulation. Recent studies have identified specific sequences in the AS promoter region that are essential for nutrient regulation (Tessmann et al., 2000). The identification of trans-acting factors that

bind these sequences, and the precise methylation status of nearby nucleotides that may influence transcription factor binding would prove informative in unraveling the sequence of events which eventually leads to AS overexpression *in vivo*. Interestingly, the Sp1 transcription factor, which is essential for both basal and amino acid induced expression of AS (Tessmann et al., 2000), is unable to bind to methylated nucleotides (Clark et al., 1997). Considering the observation by Andrulis et al., (1989) that ASNase resistant CHO cell lines had hypomethylated AS promoter regions, additional research pertaining to the relationship between Sp1 binding and AS promoter methylation in ASNase resistant cells may prove informative.

Additional data regarding the temporal order of events that leads to AS overexpression also is essential for understanding ASNase resistance. It is currently unknown whether a resistant cell is present in the initial cell population of an ALL patient, and ASNase functions to select for these "mutant" cells, or ASNase treatment somehow induces a series of genes, including AS, upon which the expression pattern may be inherited by daughter cells. Again, permanent changes in the methylation status of the affected genes would most likely account for the heritable nature of such expression patterns if this were the case.

Although the experiments in this thesis have focused on several areas of metabolism most likely to be affected by ASNase treatment, including AS, GS, and transport systems for AS substrates, it is simply not practically possible to predict or assay every possible metabolic perturbation in ASNase resistant cells using the technology employed in this study. Ideally, large-scale screening procedures which are designed to identify differences between cell lines, including differential display, gene

array blots, "gene-chip", and "protein-chip" technologies would be especially suited to identify genes that are affected by ASNase treatment. To determine the potential for these screening procedures in the analysis of ASNase resistant cells, a study employing differential display to identify potential genes involved in ASNase resistance was performed by isolating mRNA from parental and resistant MOLT-4 cells and submitting the samples to the UF ICBR differential display core for analysis. The results of this analysis indicated that genes were both up- and down-regulated in resistant cells as compared to parental cells, and included an amino acid tRNA synthetase, cell-cycle related genes, and a signaling pathway adaptor protein (Table 6-1).

Among the genes that were down-regulated in resistant cells as compared to parental control cells was human tyrosyl-tRNA synthetase. A reduction in tyrosyl-tRNA synthetase may potentially explain the increase in intracellular tyrosine in resistant cells as compared to parental cells (Table 3-2), however, the relationship between tyrosine and ASNase resistance is not currently understood. Another down-regulated mRNA identified in the differential display analysis is Cyclin I. The coordination of cell cycle control is accomplished by the activity of cyclins, along with their associated cyclin dependent kinases (CDKs), which are activated and regulated by cyclin binding. Although the concentration of CDKs remains constant throughout the cell cycle, the levels of cyclins are differentially expressed throughout the cell cycle and also may be regulated by tumor suppressor genes p53 and p21. Cyclin I is a recently identified gene that appears to share homology with Cyclins G1 and G2, although the expression of Cyclin I does not appear to be cell-cycle dependent (Nakamura et al., 1995). The function of the Cyclin G family has not been identified, however, the level of Cyclin G1

Table 6-1. Differential Display of Parental and Resistant MOLT-4 Cells. Total RNA was isolated from parental and resistant MOLT-4 cells and submitted to the UF ICBR differential display core for analysis. The indicated genes were found to be differentially regulated between the two cell lines, with parental cells serving as a basal control.

Clone	Regulation	Name	Acession Number
1K	Down	Human tyrosyl-tRNA synthetase mRNA	U89436
5A	Down	CD2 T-cell antigen	HUMANTCD2
8A	Down	Cyclin I	AF135162
9A	Up	Human mitosin	HSU30872
10E	Up	KIAA0210	D86965
11A	Up	Unknown	-
13A	Up	RalBP1-associated EH domain protein Reps1	AF031939

is induced following p53 induction, signifying that Cyclin G1 may mediate the p53 response (Okamato et al., 1999). In addition, overexpression of Cyclin G1 increases TNF-α induced apoptosis in murine cell lines, indicating that the expression of Cyclin G1 may mediate the apoptotic response (Okomato et al., 1999). Although it is not clear whether Cyclin I retains the functional similarities of Cyclin G1, in terms of ASNase resistance, the suppression of a potential pro-apoptotic factor such as Cyclin I may be essential for the proliferation of resistant cells. Clearly, the differences in apoptotic mechanisms between parental and ASNase resistant cells must be elucidated to fully understand ASNase resistance, and perhaps the use of apoptosis specific cDNA array blots to pinpoint differentially regulated genes could be helpful in understanding these complex pathways.

Among the up-regulated genes identified in the differential display analysis is mitosin, a protein that is essential for mitotic-phase progression. Mitosin is spatially regulated in the cell by phosphorylation, and condenses from a diffuse expression in S phase to specific kinetochore/centromere, spindle apparatus, and midbody localization during M-phase progression (Zhu et al., 1995). Mitosin has been proposed to function during chromosome segregation, and appears to be required for cell-cycle progression, based on the observation that overexpression of N-terminal truncated mitosin results in the accumulation of cells blockaded at G2/M (Zhu et al., 1995). Interestingly, the other up-regulated gene identified in the differential display analysis, RalBP1 associated Eps-homology domain protein (Reps1), also may have an association with cytoskeletal regulation.

Reps1 is a binding protein active in the transduction of signals via the Ras-related (Ral) family of GTPases. Ral proteins RalA, and RalB are downstream targets of Ras, and are activated by GDP-GTP exchange. Activated Ral interacts with Ral binding protein 1 (RalBP1), a GTPase activating protein that mediates downstream signaling of actin cytoskeletal components and the JNK pathway (Ridley, 1995). Reps1 has been identified by two-hybrid analysis to interact with RalBP1, and also may mediate Ral1 function (Yamaguchi et al., 1997). Also, the presence of an Eps homology (EH) domain in Reps1 indicates that this protein also may be involved in endocytosis and actin/cytoskeletal regulation, based on the presence of this motif in Eps15, a protein which promotes the association of the EGF receptor with clatharin vesicles (Fazioli et al., 1993).

Although the association between cytoskeletal rearrangement and ASNase resistance is unclear, the approach of differential display, and indeed other large-scale screening procedures have the potential to identify a disparate array of genes that may be in some way influenced by ASNase treatment. Unfortunately, the characterization of relevant genes that may prove useful in terms of drug targets to enhance ASNase therapy is a daunting task when presented with such a vast amount of information.

Ultimately, the goal of this research is to improve patient care of individuals receiving ASNase treatment. To this end, it will become necessary to accumulate data on AS expression in patient blood samples before, during, and after ASNase therapy. Currently, the mode and intensity of ASNase treatment is based on the classification of ALL by morphologic markers. Unfortunately, there is no pre-treatment prognostic indicator that may signify the potential success of ASNase treatment in selected patients.

Furthermore, due to the wide assortment of drugs used in a modern treatment regimen, it is often unclear to which drug the patient has accumulated resistance. A diagnostic test for AS expression, perhaps using an automated diagnostic platform to amplify the specific target mRNA sequence would be helpful in predicting clinical outcomes and treatment strategies.

Given that AS alone can protect cells from ASNase treatment, it follows that a gene "knockdown" strategy may be effective in rendering ASNase resistant clinical cases sensitive to ASNase therapy. A gene therapy approach using a transcription factor decoy vector could prevent the up-regulation of AS in ASNase resistant cells by competitively inhibiting specific transcription factors essential for amino acid-induced transcription. Also, a direct inhibitor of the AS enzyme would be a useful theraputic tool. Perhaps the most effective use of a knockdown strategy may be during the initial remission induction phase of treatment. Because the expression of AS blocks ASNase-induced cytotoxicity (Figures 3-9, 3-10), it follows that a course of ASNase treatment in the presence of an AS transcription inhibiting agent may result in a potent synergistic remission induction regimen.

In addition to the use of AS as a diagnostic marker, the data regarding System A may be relevant in terms of a potential treatment for ALL. The observation that parental cells are unaffected by a saturating concentration of the specific System A inhibitor MeAIB, whereas resistant cells undergoing ASNase treatment become more susceptible to the drug in the presence of MeAIB, indicates that this compound may be useful to enhance ASNase therapy. Clearly, *in vivo* studies are required to determine the system-wide effects of MeAIB treatment, as well as the potential anti-tumor effectiveness in a

whole organism, however, an inhibitor which appears to have such a specific potentiating effect on ASNase treatment may be worth further investigation.

In conclusion, this work has documented the expression of amino acid transporters, amino acid synthesizing enzymes, and signaling pathway intermediates in response to ASNase treatment. Also, differences between parental and ASNase-resistant cell lines were identified that may be useful as potential targets for future therapeutic approaches. Certainly, using the tools of modern molecular biology, far more is known about ASNase than when it was introduced as a therapy in 1930. To utilize this knowledge, a much more comprehensive analysis of drug resistant cells *in vivo*, as well as additional study on the regulation of the AS gene itself must be performed to fully understand the effects of ASNase treatment and transfer this basic research to a clinically relevant approach to the treatment of ALL

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BIOGRAPHICAL SKETCH

Ara Aslanian was born on February 17, 1971 in Newton Falls, Massachusetts and was raised at 210 William G. Drive, Tewksbury, Massachusetts. He attended Central Catholic High School in Lawrence, Massachusetts, during which time he obtained a private pilot's license. Ara continued his education at the University of Massachusetts, Amherst, and was awarded a degree in Biochemistry and Molecular Biology in 1994. After graduation, he was employed at Gene-Trak systems as an associate scientist, where he worked on projects involving nucleic acid hybridization-based medical diagnostic tests. In the summer of 1995, Ara entered the doctoral program in Biochemistry and Molecular Biology at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael S. Kilberg, Chair
Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry S. Nick
Professor of Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter M. McGuire
Associate Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald A. Novak
Professor of Pediatrics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sheldon M. Schuster
Professor of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dean, College of Medicine

Dean, Graduate School